CHAPTER

Thinking Transgenic Vectors in a Population Context:

Some Expectations and Many Open-Questions

Christine Chevillon,* Richard E. Paul, Thierry de Meeûs and François Renaud

Abstract

The present papers tries to place questions regarding the eventual release of transgenic *Plasmodium*-resistant mosquitoes within an overall population context. This means a context that is not limited to selection outcomes regarding the resistance/susceptibility of the *Anopheles* targeted by transgenesis, but that also recovers selection outcomes regarding other traits, demographic outcomes (via the possible number of transgenic mosquitoes to release), and bio-diversity outcomes regarding the vectors and parasites that cocirculate in the chosen release locality. By considering all these outcomes together, we highlight missing biological data necessary for any correct quantitative evaluation of the probability of success of a transgenic release. Qualitative evaluations are nonetheless possible to perform: they suggest that there is a very weak probability for released transgenic mosquitoes to actually succeed in modifying malaria transmission. However, the main interest of the present discussion does not concern these qualitative conclusions. Instead, it highlights the necessary biological knowledge of malaria for a correct evaluation of the fate and consequences of eventual releases of transgenic vectors, and more generally of the evolutionary possibilities and constraints of any change in transmission characteristics. As such, we hope that the present discussion underlines the necessity to address new fundamental questions regarding malaria biology in order to actually capture the mechanics regulating the evolutionary dynamics of *Plasmodium* burdens and associated pathologies.

From the outset, it is assumed that mosquito trans-genesis, inducing *Anopheles* resistance to *Plasmodium* infection, becomes sufficiently "routine" such that the major issue would concern the choice of the candidate to release in order to maximize public health benefit. From then, we explore the extent to which concepts of population genetics and evolutionary biology may help in evaluating, or even optimizing, the chance of a successful transgenic release strategy. One approach would be to develop explicit mathematical models targeting the epidemiological consequences of the vector evolution toward parasite-resistance. This is fruitful[1] but requires *a priori* assumptions, concerning the estimates taken by several population genetics parameters, whose biological pertinence may be difficult to evaluate. Thus, we chose an alternative approach with the hope of strengthening trans-disciplinary discussions regarding the practical

*Corresponding Author: Christine Chevillon—Team 'Evolution des Systèmes Symbiotiques', Laboratoire G.E.M.I. (UMR IRD-CNRS 2724), Centre IRD, 911 avenue Agropolis BP 64501, F 34 394 Montpellier cedex 5, France. Email: christine.chevillon@mpl.ird.fr

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constraints linking transgenic production to release. Therefore, we have tried (i) to clearly define the population genetics parameters that both determine the fate of transgenic mosquitoes in natural populations and are easy to measure in experimental set-ups; (ii) to make explicit the assumptions hidden behind the structure of population genetics models, and (iii) to confront assumptions with biological data. Finally, where knowledge is lacking, we have tried to underline the neglected biological questions that would provide invaluable information.

This framework is applied to three complementary issues that delimit the sections of the present chapter. The first section is aimed at identifying the early risks of trans-gene disappearance and the precautions to take in order to minimize these risks. The second section defines the parameters, accessible to experimental evaluation, that determine the long-term evolution of the released resistance trans-gene and their epidemiological consequences under idealized conditions. We have also tried to show how incorporation of increasing levels of biological reality may alter conclusions. This enables clear identification of the biological data necessary for a correct evaluation of the epidemiological consequences of any release of *Plasmodium*-resistant mosquitoes. Such requisite data will be confronted with the current knowledge on malaria biology in a third and final section, exposing several gaps between required and acquired knowledge. Overall, this discussion hopes to highlight, not only the reasons why evolutionary biologists are so skeptic about the public health benefits to expect from transgenic resistance release, but also the field and experimental studies that we must address in order to understand the mechanics regulating the evolutionary dynamics of *Plasmodium* burdens and associated pathologies.

Transgenic Naturalization—Considerations for Successful Invasion

It was claimed that *'first transgenics released must be sterile'*.[2] As a preliminary argument, let us wipe out a possible confusion between two mutually exclusive processes aimed at modifying the epidemiology of vector-borne diseases. One strategy was the release of sterile vector males (possibly resulting from transgenesis, see ref. 2 for review). These sterile males have been hoped to reduce vector demography through competition between sterile and wild males for wild females. For this strategy to have an impact, humans should produce and release, at each mosquito generation, a number of sterile males that matches that of the wild-type females seeking a mate, or even higher number if females can have several mates; *An. gambiae* females mate more than once,[3] and frequently occur in large numbers within populations. Thus, it is not surprising that this 'sterile male' strategy failed when applied to this species complex.[4]

Examining the potential of releasing *Plasmodium*-resistant mosquitoes radically changes the rationale. Indeed, a trans-gene that confers resistance to *Plasmodium* infection can be effective if and only if it is designed to be expressed by female mosquitoes blood-feeding on humans. This is because only female mosquitoes face infection and they do so while blood-feeding. Therefore, whenever one considers such a strategy to improve public health, he/she automatically considers releasing transgenic mosquitoes, of either sex, that will lay fecund transgenic female descents in nature. If modifying the genetic composition of a vector population and letting people being bitten by transgenic females raise ethical concerns,[2] it is noteworthy that these concerns are actually inherent to any success using a *Plasmodium*-resistance release.

But what does determine the probability of success of such a strategy? It depends on the relative fitness of transgenic mosquitoes and on that of the trans-gene itself. The fitness of transgenic relative to wild-type mosquitoes can be measured by their average difference in offspring number. The fitness of the trans-gene corresponds to the average number of copies generated per generation relative to the equivalent average computed for a standard nonselected ('neutral') gene. Equivalence between these fitnesses only occurs when trans-gene carriers share the same genetic background as wild-type mosquitoes. This condition characterizes what we call a 'naturalized trans-gene' (Fig. 1A). Thus, the process clearly discriminates a prenaturalization period, when the success of the transgenic strategy is almost independent of trans-gene fitness, and a post-naturalization period when success is tightly linked to trans-gene fitness. The case of post-naturalization period will be discussed in the section "Evaluating the Chance of Success of Naturalized Resistance Genes: Formalization and Estimation of the Selective Balance Involved".

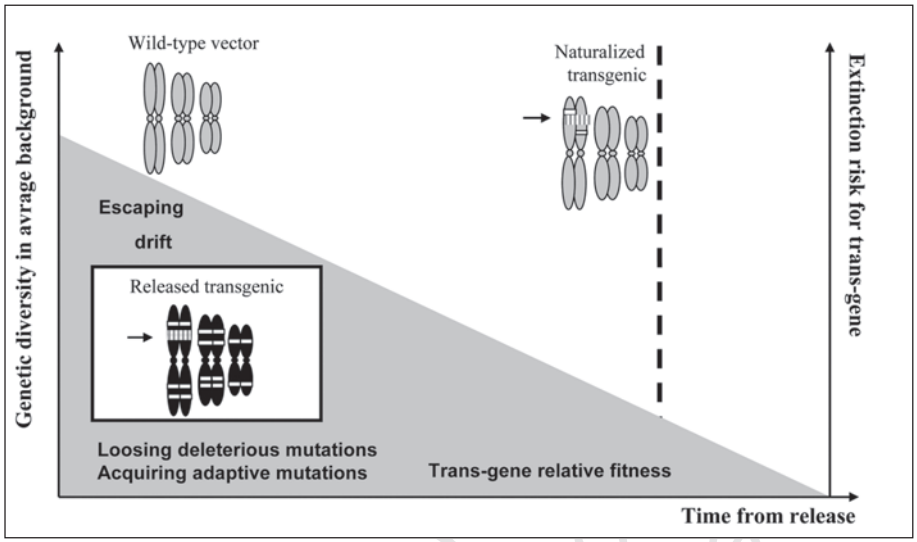

Figure 1A. The dangers faced by laboratory-selected genes when released in populations: intensities, causes and protection means. At release time, a *Plasmodium* resistance-gene (figured here as a locus pointed by an arrow) is necessarily borne by a laboratory genetic background. This laboratory genetic background is likely to be on average more inbred and to bear more homozygous deleterious mutations that the average wild-type one (see text). We draw here deleterious mutations as white loci, genetic backgrounds of low diversity as black chromosomes, of high diversity as grey chromosomes, intermediate diversity levels as chromosomes bearing black and grey motives, with in this latter case identify/difference in motives reflecting genetic identity/differences. Figure 1A pictures the differences regarding the genetic backgrounds of a wild-type vector and of a transgenic mosquito taken either at release time or at the end of the naturalization period (dotted line). The grey area illustrates the time-evolution of extinction risks for the trans-gene. The sources of extinction risk are indicated in bold characters with reference to their relative time of predominance. Please note that the highest risks occur very early after the release time and that the trans-gene fitness will only become important near the end of the naturalization period.

Drift, Inbreeding and Background Selection: The Three Major Risks in Prenaturalization

The extinction risks that the trans-gene faces before it achieves naturalization originate from three properties of any laboratory-strain release (Fig. 1A). First, the relative frequency of the released transgenic mosquitoes compared to wild-type ones will be relatively low. This not only opens the road for genetic drift to accidentally clear the recipient population from any trans-gene carrier, but also to do it rapidly and independently of any fitness consideration.

Even if enough mosquitoes are released to escape the immediate risk of drift, two other dangers are likely to arise because of the higher inbreeding expected among laboratory-released compared to wild-type mosquitoes. The released transgenic mosquitoes are descended from one or a few strains that have evolved for many generations under laboratory conditions. Accordingly, genetic drift and selection for adaptation to laboratory environments are expected to have (i) progressively increased the genetic divergence between laboratory-descendents and wild ancestors at many loci (including those that can be advantageous in the environment of release), (ii) progressively reduced the genetic diversity of laboratory descendents down through the generations, and (iii) fixed by chance a few deleterious mutations into the genetic background of these laboratory descendents. Therefore, for the transgene to have a chance to persist within the recipient population, the associated genetic backgrounds must lose their deleterious mutations and acquire the locally adaptive alleles. Two complimentary experimental axes had confirmed

the reality of these theoretical risks: the first refers to ecosystem restoration, the second to laboratory investigations of competition between transgenic and wild-type mosquitoes.

Experimental Lessons from Ecosystem Restoration

The release of transgenic mosquitoes into a totally new environment already occupied by wild vectors resembles the translocation of organisms performed in attempts to restore native ecosystems. Local attempts of ecosystem restoration have been regularly performed by attempting to settle immigrants into endangered populations. This thus provides a rather global experience regarding the probability and conditions of success and failure.[5]

Experience has shown that failures, or at least strong difficulties for immigrants to settle, are the overriding outcome.[5] Interestingly, the exceptional cases of success occurred either when the number of native individuals was small compared to that of the transplanted immigrants, or when the native population displayed low genetic diversity.[5] Unfortunately, neither of these two scenarios can ever reasonably be expected to apply to the release of transgenic mosquitoes. Numerical dominance of a vector population by laboratory-produced mosquitoes is almost inconceivable. It is also highly improbable that the natural vector population targeted would have suddenly experienced a drastic loss in genetic variability just before the release of transgenic vectors.

By contrast, the difficulties recurrently encountered by immigrants in ecosystem restorations are very likely to apply to the release of transgenic mosquitoes. A first difficulty arises because the transplanted immigrants bear genotypes that confer adaptation to their ancestral but not to their new environments.[5,6] Given the large differences between laboratory-controlled and field environments, it is very likely that such mal-adaptation will also concern the laboratory-engineered mosquitoes at release time. A second difficulty arises because immigrants display too low a genetic diversity and too much inbreeding to get rid of their deleterious mutations within their new challenging environment.[5] Avoidance of inbreeding is a difficult goal to achieve in laboratory-reared strains, requiring either the application of laborious mating protocols or regular incorporation of individuals from foreign stocks. Therefore, this second difficulty is also very likely to apply to released transgenic mosquitoes, and indeed has been directly confirmed in laboratory population experiments.

Trans-Gene Naturalization in Laboratory Experiments: The Actual Dimension of Inbreeding

Trans-genesis is rarely successful at 100% so that transgenic strains are generally founded by a very few individuals. Therefore, the risk for a chance fixation of deleterious mutations looks even greater within transgenic than in standard laboratory strains. A recent study investigated this question by allowing transgenic and wild-type mosquitoes to compete within experimental populations maintained under laboratory conditions (to which all competitors were adapted).[7] Four transgenic lines of *Anopheles stephensi* were involved where trans-genes encoded distinct fluorescent proteins. Genetic drift was avoided by seeding populations with a 50:50 mix of transgenic and nontransgenic mosquitoes. In all replicates and whatever the identity of the trans-gene, the outcome was disappearance of the trans-gene from experimental populations within a few generations. Making the wild-type mosquitoes as inbred as the transgenic ones was enough to considerably increase the number of generations during which the trans-gene persisted.[7]

A later study reinvestigated this issue by focusing on two transgenic constructs that make *Anopheles* mosquitoes resistant to *Plasmodium* infection.[8] A first transgenic construct encodes a tetramer of SM1 peptide. This peptide prevents vector infection by competitively binding to the *Anopheles* receptors that *Plasmodium* parasites use to infect the vector.[9] In this case, homogenization in genetic background among competing mosquitoes was sufficient to ensure trans-gene naturalization in all replicates originally seeded with half wild-type and half transgenic mosquitoes.[8] However, the conclusion was different for a construct encoding the bee venom protein PLA2, which prevents *Plasmodium* infection through an unknown mechanism.[10] This trans-gene

consistently disappeared in five generations, even after having homogenized the genetic background among competitors. This indicates that the presence of the PLA-2 trans-gene deteriorates mosquito fitness. Whether this counter-selection was due to PLA2 production or to the chromosomal localization of trans-gene insertion remains to be clarified.[8]

Overall, these studies confirmed that trans-gene naturalization is a difficult goal to achieve in *Anopheles* strains even when the effects of genetic drift and of environmental changes are avoided. In these optimal conditions, the major and inescapable source of difficulty stems from the high inbreeding observed within transgenic lines.

Solutions to Naturalization Problems

Sorting Out the Least Costly Trans-Genes: The Beginning of a Real Solution

Studies on the genetics of adaptation have recurrently shown that mutations generally tend to decrease fitness when expressed in new genetic backgrounds and/or new environments, but that such a fitness cost varies so greatly among mutants than it can occasionally be null.[11-15] Thus, we can reasonably anticipate a variation in fitness cost among trans-genes whether this cost arises from the expression of the trans-gene or from the trans-genesis process. As a consequence, common sense recommends increasing the range of mutants to incorporate into trans-genesis protocols in order to pick up those associated with the weakest fitness cost. Nonetheless, it is noteworthy that the question of fitness cost intensity per se is far from being the most crucial for the fate of the 'transgenic strategy'. This issue will be discussed in details in the section "Evaluating the Chance of Success of Naturalized Resistance Genes: Formalization and Estimation of the Selective Balance Involved".

Diversifying the Genetic Background of Transgenic Strains: An Achievable Requirement

Although the necessity of using out-bred strains to engineer transgenic mosquitoes has been recognized,[7] genetic drift and low numbers of reproducing adults are so difficult to avoid that an originally out-bred strain is very likely to generate inbred transgenic mosquitoes at release time. A partial solution to alleviate this problem would be to introduce foreign genotypes into transgenic homozygotes just before release time. This may be further optimized if the foreign genotypes are picked up from the vector population targeted by the release strategy. Indeed, such a process will tend to optimize the probability of incorporating the locally adaptive genes into the background of the homozygous transgenic mosquitoes. Replicating such an introgression strategy with parallel population-cages would incorporate alternative wild-type genotypes in the background of the transgenic mosquitoes (Fig. 1B). A final cross among the descendents of these introgressed populations will further diversify the wild-type genotypes of transgenic mosquitoes (Fig. 1B). This crossing protocol looks *a priori* as the most efficient in minimizing the predicted naturalization problems and even reducing the high risk period of naturalization (Fig. 1C). This was indeed one conclusion from an experimental test for the potential of plant population restoration.[6]

Counting on Genetic Drive to Shorten the High Risk Naturalization Period: An Impossible Dream?

The possibility to reduce the critical period of trans-gene naturalization with the help of genetic drive has received a lot of attention.[1,16-21] A genetic element promoting genetic drive tends to be over-represented in the crossed descendents of carrier and noncarrier parents. Two drive systems have been considered: *Wolbachia*-borne and transposon-borne trans-genes. *Wolbachia* are bacteria that parasitize the cells of many Arthropods, have maternal inheritance, and that profoundly affect the reproduction of their host. For instance, *Wolbachia pipientis* affects the fertility of *Culex* and *Aedes* mosquitoes as follows: (i) crosses between *Wolbachia*-infected females and noninfected males are fertile, (ii) crosses between *Wolbachia*-infected males and noninfected females are sterile, and (iii) crosses between two *Wolbachia*-infected parents have variable

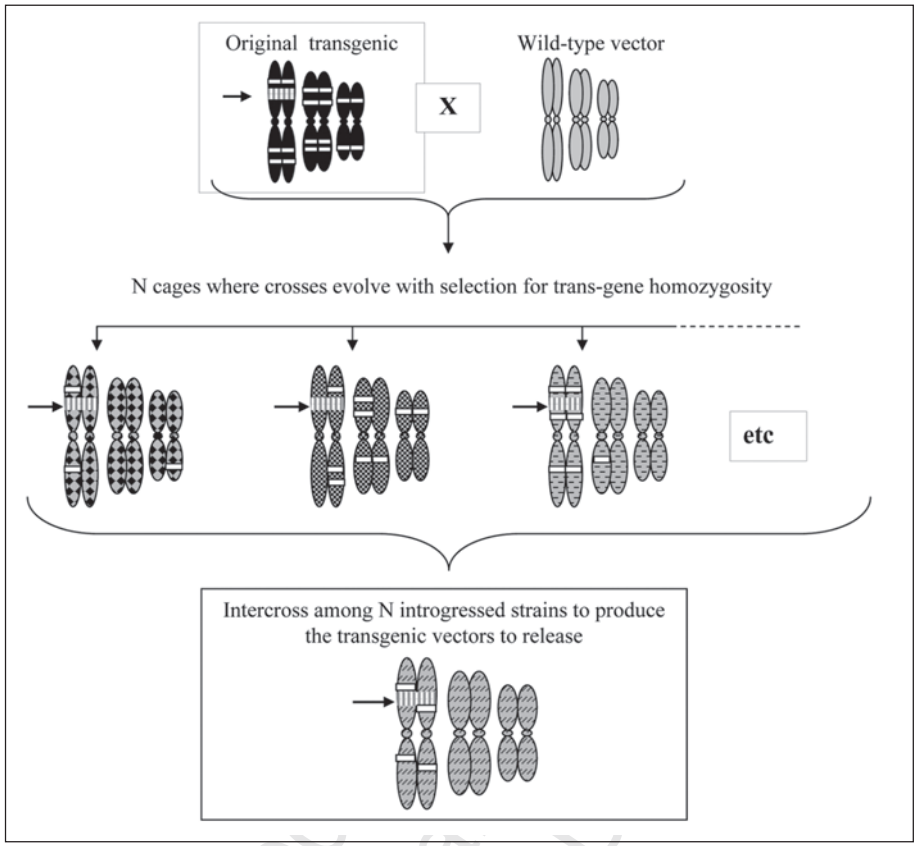

Figure 1B. Using the color conventions of Figure 1A, Figure 1B describes a crossing protocol to apply between the production and release times of *Plasmodium*-resistant mosquitoes. Each population-cage is initially seeded by the offspring of the originally produced transgenic mosquitoes crossed to individuals captured in the population targeted by the future release. Free evolution is allowed in these cages except that only homozygotes for the transgene are allowed to mate. As a consequence, each population will experience a different history and will select for a different genetic background than others. A final cross among resulting strains will thus reincrease the genetic diversity and help remove the remainder deleterious mutations.

outcomes depending on the genetic relationships of the bacteria involved. As *Anopheles* mosquitoes are seemingly *Wolbachia*-free in natural populations, the use of a *Wolbachia* from *Culex* or *Aedes* to bear a resistance trans-gene has been proposed with the idea that the resulting transgenic females will be able to mate with any wild-type male and to transmit the trans-gene to almost all resulting offspring.[16] However, mathematical analyses showed the occurrence of stringent conditions for *Wolbachia*-induced incompatibilities to actually be able to drive *Wolbachia*-borne trans-genes in populations.[17-19] Moreover, if all *Wolbachia* effects remained unchanged in *Anopheles* host, then transgenic males would never be able to produce any viable progeny when mating to wild-females. This poses a serious problem for achieving the necessary reduction in the inbreeding of released transgenic mosquitoes; therefore a reduction in prenaturalization period by such a *Wolbachia*-strategy seems very unlikely.

Alternatively, as insect trans-genesis generally uses transposon elements,[8,9,22-24] genetic drive via transposon elements was hoped to shorten the naturalization period and to reduce the associated risks of trans-gene disappearance.[1,20,21] However, a review has recently reduced these

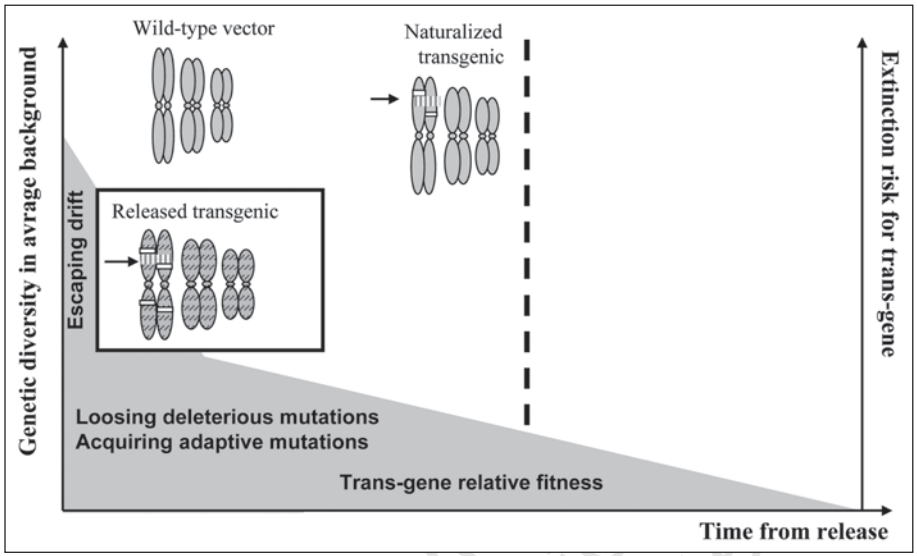

Figure 1C. Using the same conventions as Figure 1A, Figure 1C describes the time-evolution in extinction risks that would be experienced by the introgressed transgenic mosquitoes (obtained from Fig. 1B) once released in the field. Note that the main effect of the crossing protocol would be to reduce the length of the risky period of prenaturalization.

hopes to zero by showing that the used transposon elements are at best very poorly remobilized within mosquito genomes.[24]

Evaluating the Chance of Success of Naturalized Resistance Genes: Formalization and Estimation of the Selective Balance Involved

Hereon, we ignore the differences in genetic background between transgenic and native vectors in order to dissect the processes that define the relative fitness of the resistance trans-gene, the evolution of the resistance phenotype and the epidemiological consequences of this evolution. This section is written with the aim of stimulating discussion among all potential actors involved in a transgenic release project whether they are concerned with laboratory processing, field studies, public health surveys or political decisions. We have thus avoided refining the mathematical models previously developed.[1] Instead, we argue on the biological reality of parameters, their accessibility to estimation in real life, and on their qualitative influence on the conclusions. Therefore, we have tried as much as possible to highlight the bridges that should connect the experimental domain- aimed at identifying and/or modifying the mechanics of *Anopheles-Plasmodium* interactions—to the world in silico—where mathematical models try to capture the evolutionary dynamics of the *'Homo-Anopheles-Plasmodium'* system.

From Biology to Minimal Formalization Able to Forecast Resistance Evolution

The presence and the absence of the risk of *Plasmodium* infection describe two qualitatively distinct environments referred to by the indices I and NI, respectively. The susceptibility and resistance to *Plasmodium* infection define the S and R phenotypes, respectively. Finally, W_{XY} refers to the mean fitness realized by the X phenotype within the Y environment. Given the lack of knowledge on resistance pleiotropy, let us simply assume here that only female fitness is concerned. Regarding resistance evolution, the question of interest is to formalize the overall selective balance acting on R and S phenotypes across I and NI environments.

It should be noted that various environmental variations will surely affect vector fitness (temperature, availability and quality in food resources, competition, infection by other pathogens, predation etc). Accordingly, no pertinent predictions can be made from estimates of W_{SNI}, W_{SI}, W_{RNI} and W_{RI} that were measured in independent set-ups and hence very likely to be differently biased by confounding environmental variations. Therefore, one solution is to move one-step backward, by focusing on the direct fitness comparison of S and R mosquitoes that are conjointly experiencing the exact same environment rather than the W_{XY} components themselves. This is the rationale hidden behind the formalization using *c* and *s* parameters: these parameters are measuring R-to-S differences in fitness within either one of two alternative environments. As such, population cage experiments where R and S are competing a few generations long (as those reported in refs. 7,8) are accurate to estimate the average value taken by the resistance fitness cost *c*. The parallel experiment in which infectious blood-meals are given to the competitive vectors would allow correct estimation of the average fitness advantage of resistance *s*. Please note that the *s* estimate tightly depends on the I environment where it is measured: a different choice in the environmental reference is likely to lead to a different estimate. From then, it is noteworthy this I environment is also defined with reference to NI environment through the parameter *d*; i.e., the average detriment in fitness that infectious contact imposes to susceptible mosquitoes. This precision is important because it underlines that (i) any *s* estimate indirectly depends on the joint *d* estimate, and (ii) the arising function $s_{(d)}$ emerges as an inherent property of the environmental conditions chosen as references (i.e., averages in human-blood composition and abiotic parameters, but also in blood-meal concentration in *Plasmodium* gametocytes, parasite genetic composition, and gametocyte infectiousness etc). Therefore, any erroneous appreciation regarding the environmental range experienced by susceptible vectors and the associated variations in vector fitness and/or in parasite fitness would define major sources of errors regarding the estimation and evolutionary importance of the fitness advantage of resistance. Hereafter, the notation $s_{(d)}$ will replace the notation *s* whenever an evolution in I and NI environmental references is suspected.

For the moment, let us remain with fixed environmental references and describe the formalization using the associated estimates of *d*, *s* and *c* parameters (Fig. 2A). Parameter *d* measures the fitness detriment that may be imposed by *Plasmodium* infection upon susceptible vectors: $W_{SI} \leq W_{SNI}$ and $W_{SI} = (1\text{-}d).W_{SNI}$ with $0 \leq d \leq 1$. Parameter *s* measures the fitness advantage that resistance confers when a blood-meal is infectious by its infection-blocking effect; thus $W_{RI} = (1 + s).W_{SI}$; with $s \geq 0$. Parameter *c* measures the fitness cost of resistance when infection risk is absent; thus $W_{RNI} = (1\text{-}c).W_{SNI}$ with $0 \leq c \leq 1$. Noting *f* the probability for a vector to face the risk of *Plasmodium*-infection (i.e., to ingest gametocytes, the parasite stages that are infectious to vectors), the overall selective balance acting on the R/S polymorphism across I and NI environments is simply given by:

$$W_R - W_S = f.\,[W_{RI} - W_{SI}] + (1\text{-}f).\,[W_{RNI} - W_{SNI}] \quad (1)$$

The pair-wise relationships among female fitness components lead then to rewrite this equation as:

$$W_R - W_S = W_{SNI}.\,[f.s.(1\text{-}d) - (1\text{-}f).c] \quad (2)$$

Accordingly, the constraints under which resistance and susceptible phenotypes freely coexist within the local recipient population (i.e., $W_R = W_S$) are defined by:

$$f.s.(1\text{-}d) = (1\text{-}f).c \quad (3)$$

Alternatively, as transgenic resistant vectors are aimed to be released in viable vector populations, where $W_{SNI} > 0$, the overall fitness difference $[W_R - W_S]$ will have the same sign as the expression $[f.s.(1\text{-}d) - (1\text{-}f).c]$. Thus, the resistance phenotype will be allowed to locally increase in frequency if and only if $f.s.(1\text{-}d) > (1\text{-}f).c$.

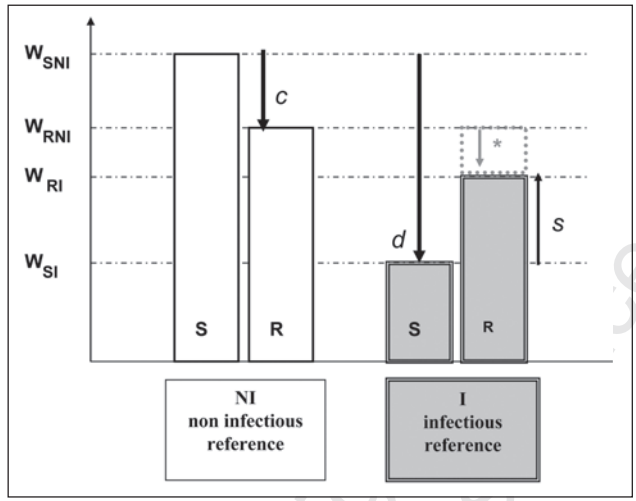

Figure 2A. Fitness effects that are directly induced by the resistance gene: formalization, estimation and evolutionary consequences. Given two environmental references for the vectors, depicting respectively parasite-free (NI) and infectious (I) conditions, this figure pictures a possible outcome for the R-to-S comparison in fitness performance. The fitness detrimental effect of parasite infection in susceptible vectors (pictured by the *d*-indexed arrow) is an emerging property of this pair of environmental references. From then, fitness comparisons among phenotypes are computed within environments. This allows the most pertinent evaluation of the fitness resistance cost (the arrow indexed by *c* letter) and fitness resistance advantage (the arrow indexed by *s* letter), as any confounding environmental variation will simultaneously affect S and R performances. Here, the grey arrow (indexed by a star) illustrates the so-called physiological cost of resistance that is too often confused with that of the resistance fitness cost. Please note that the main effects of this physiological cost are to define a fitness decrease for resistant phenotype in I environment relative to NI, or equivalently to impose an upper-limit to the fitness advantage of resistance. Whenever I and NI environmental references accurately capture the field-environmental variation in selection, a correct evaluation of $\alpha = c/s$ will help forecaste resistance evolution.

Biological Consequences on the Minimal Requirements for Resistance Evolution

The first requirement is obvious: if there is no fitness advantage to resistance ($s = 0$), there must be no fitness resistance cost for the resistance phenotype to be able to persist! In this case, $s = c = 0$: the success of transgenic release strategy depends on the fate of an introduced neutral phenotype, hence on its extinction probability through drift. Both phenotypes would have an average fitness of $W_{RNI} = W_{SNI}$ in parasite-free environments, and of $W_{RI} = W_{SI} = (1\text{-}d).W_{SNI} = (1\text{-}d).W_{RNI}$ when facing infectious contacts. Interestingly, the value taken by *d* does not need to be null and estimates two independent quantities at once. In susceptible vectors, it goes on estimating the fitness detriment caused by parasite development, i.e., the effect of virulence of the local *Plasmodium* parasites toward their local vectors. In resistant mosquitoes, because of the emerging relation $W_{RI} = (1\text{-}d).W_{RNI}$, the value taken by *d* quantifies the fitness detriment imposed by the physiological changes allowing them to block parasite development. In other words, here, *d* measures the physiological cost of a selectively neutral resistance trans-gene! This highlights once more the absence of synonymy between the physiological and the fitness costs of resistance.[25,26]

Now, it is noteworthy that assuming $d = 0$ does imply $s = 0$ (hence, as above, this implies that resistance evolution relies on the hazardous introduction of a selectively neutral phenotype). Indeed, what can be the fitness advantage of blocking *Plasmodium* development if this infection does not impair fitness? This is a major reason why questions regarding the potential virulence of *Plasmodium* parasite towards their vectors are so crucial.

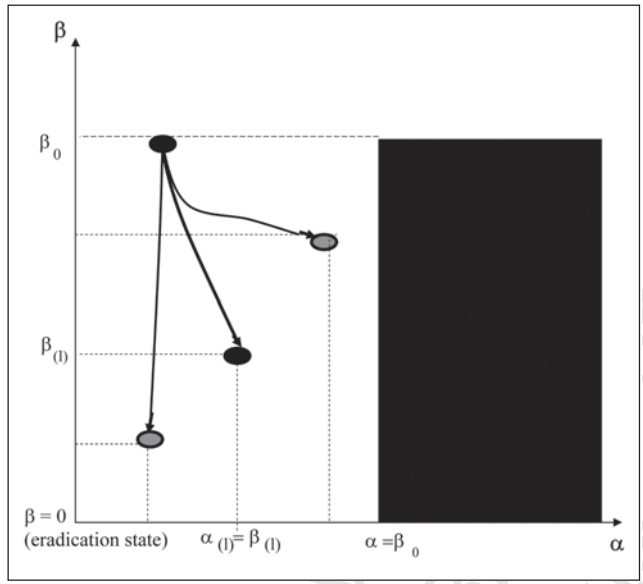

Figure 2B. This figure pictures the conditions of resistance evolution in $\alpha_x \beta$ space. Given the initial values β_0 and α_0, resistance will be allowed to evolve as soon as $\alpha_o < \beta_o$ (i.e., in any point located outside the black-colored area). From then, the expected reduction in human gametocyte burden following the vector evolution of resistance (hence the reduction in *Plasmodium* transmission) will tend to decrease β. Meanwhile, the epidemiological changes induced by vector evolution are very likely to change the average quality of infectious environments (e.g., through changes in human distribution in gametocyte loads and/or genetic composition). In addition, as vector evolution toward parasite resistance will lead to an increase in vectors heterozygous for the trans-gene, it will change the average response to infection among the contemporaneous phenotypes that are determining the phenotypic references for resistance and susceptibility. As a consequence, the evolution of vector resistance is expected to jointly modify the future values taken by *d* and *s* parameters, and hence by α. In other words, the evolution of resistance in a vector population creates the conditions for a coupled-evolution of α and β. A precise formalization of these α-β coupling-rules will require knowing the functions that determine (i) the dependence of gametocyte load distribution on the probability for human to be infected, (ii) the dependence of the d parameter on human gametocyte load, and (iii) the dominance of s and c parameters in heterozygous vectors. In the absence of this required knowledge, only qualitative conclusions can be made regarding α-β coevolution. In this figure, three qualitative possibilities are pictured with evolution-time following the sense indicated by arrows and evolution starting at the same initial state (α_0; β_0). They are ranked accordingly to the degree of dependence between α and β along their evolutionary course: α and β are rather uncoupled in the example figuring on the left, but evolve in tight interdependence in that figuring on the right. These differences in α -β interdependence did not prevent the occurrence of a time limit l where the whole system reaches an equilibrium defined by $\alpha(l) = \beta(l)$ and $\beta(l) > 0$ (i.e., an absence of parasite eradication), but do nevertheless modify the equilibrium identity through its characteristics proportions in infected humans and vectors.

Finally, let us examine the cases where *Plasmodium* infection does impair the fitness of susceptible vectors ($d > 0$) and resistance does confer a fitness advantage to mosquitoes facing infection ($s > 0$). An extreme case occurs when local humans are all bearing *Plasmodium* gametocytes, such that any blood-meal is infectious for vectors: $f = 1$. In such a case, vector resistance to *Plasmodium* will initially increase in frequency whatever the values taken by *c*, *s* and *d* parameters. Otherwise ($0 < f < 1$), the fate of resistance depends on the comparison between two ratios: $\alpha_{(d)} = c/s_{(d)}$ and $\beta = [f(1\text{-}d)] / [1\text{-}f]$. An overall selective advantage will favor resistance whenever $\alpha_{(d)} < \beta$, and susceptibility whenever $\alpha_{(d)} > \beta$. One advantage of this formalization is to separate what is specifically related to the fitness comparison among phenotypes within the

two chosen environments, from what is inherent to the targeted population. Moreover, this allows the recovery of a classical result in the genetics of adaptation:[27] it is not its fitness cost per se that matters in the evolution of a new phenotype but the suitability between the cost-benefit balance it confers across two given environments of reference ($\alpha_{(d)}$), and the relative importance of those environments both in terms of frequencies (i.e., *f* terms in β) and of the induced fitness variation in the ancestral phenotype (i.e., *d* term in β). Finally, this formalization underlines the mechanics of a feed-back linking the evolution of *Plasmodium*-resistance in mosquitoes to the conditions determining its subsequent evolution. Indeed, any increase in *Plasmodium*-resistance in mosquitoes will decrease *f*- the human-burden of *Plasmodium* gametocytes - and hence necessarily affects the future value taken by β. Complementarily, changes in the quality of the infectious environment are very likely to modify the fitness consequences of *Plasmodium* infection, i.e., to jointly modify the values taken by *d*, by the relative fitness advantage of resistance $s_{(d)}$., and hence that taken by $\alpha_{(d)}$. In summary, changes in the frequency of resistant vectors will not only modify the transmission patterns from vectors to humans but also the human-environmental referential that counts for the determination of β, *d*, $s_{(d)}$, $\alpha_{(d)}$ and hence the future issue of α-to β comparisons. This is enough to speculate that- as long as the local parasites do not evolve- the general tendency would be the occurrence of a date-limit *l,* at which time the dynamics attain equilibrium where α equals β (Fig. 2B).

Returning to real life, the interesting question would be to determine whether or not this equilibrium limit defines a good protection for public health. No pertinent answer can be provided without additional data that explicitly addresses the relationships between the human-distributions in malaria pathologies, in the overall *Plasmodium* burden and in the gametocyte burden, as well as between these human-distributions and the fitness outcomes of parasite-vector interactions (i.e., parasite effects on the fitness of susceptible vectors and the relative benefit of being a resistant vector). In parallel, the overall picture is likely to be modified by parasite evolution. To be favourable for parasite fitness, this evolution should counterbalance the decrease in human-to-vector transmission that has been caused by resistance evolution in vector populations. Among possible evolutionary answers, one solution could be for the parasite to lengthen the time during which successfully infected vectors are infectious for humans, so that a reduction in the density of susceptible vectors would not alter the local parasite density. Another evolutionary solution would be for the parasite to increase its gametocyte burdens in humans, and hence the likelihood for any infected human to participate in the human-to-vector transmission. Other evolutionary answers of the parasite can be proposed. In all cases, the message emerging here is the necessity to fully characterise the interactive relationships that a population of *Plasmodium* parasites is jointly entertaining with their human-hosts and vectors if we want to be able to evaluate (i) the relative chance for parasite evolutionary answers to vector resistance to be selected for, and (ii) the public health consequences of such potential parasite evolution. To date, required data for such an evaluation are still missing.

Confrontation with an Explicit Model Regarding Resistance Evolution within an Isolated Infinite Population

Boëte and Koella[1] developed an explicit model that was based on the following premises:

1. No parasite evolution;
2. No parasite differences in transmission or in virulence toward mosquito (i.e., each individual parasite that develops within a vector decreases its fitness by a fixed amount);
3. An even distribution in gametocyte burden among the humans infected by Plasmodium parasites at any time t;
4. A frequency of infected humans given by f (t)= [Ro(t)-1]/ [Ro(t) =a/m]; with a and m representing the mosquito's biting and mortality rates, respectively;
5. Eventuality of partial or total blockage in parasite developments in RR mosquitoes;
6. Poisson distributions in the cumulative parasite burden along mosquito life with similar parasite-dose dependent effects on the fitness detriment of mosquitoes among SS, RS and RR genotypes;

7. A fitness advantage of resistance that arises from an average reduction in parasite burden of RR and RS relatively to SS mosquitoes; so that this advantage also emerges as parasite-dose dependent;
8. RR individuals that either suffer from a fitness resistance cost, or that experience a reduction in their fitness advantage;
9. Equality in dominance levels of the resistance fitness advantage and of the resistance fitness cost;
10. The eventual benefit of genetic drive for resistance trans-gene.

The conclusions driven by this explicit model are totally congruent with those of our crude and qualitative model. Indeed, in the absence of genetic drive, all parameter combinations result in the occurrence of thresholds in the fitness cost values that allow the evolution of resistance, a diminishing return acting on any evolving resistance, and hence for the very weak probability of *Plasmodium* eradication through modification of the vectorial capacity of a local *Anopheles* population. In addition, this explicit model indicates that only perfect resistance (i.e., so that no single parasite is able to develop within RR mosquitoes) has got a nonnull probability of succeeding in a local eradication of *Plasmodium*. From then, the eventual occurrence of genetic drive may facilitate malaria eradication within this isolated population by annihilating the diminishing return effect (i.e., by uncoupling the evolution of α and β).

Deviations Caused by the Co-Ocurrence of Two Vector Species

The very effect of the co-occurrence of two vector species is to tend to uncouple the local evolution of *Plasmodium*-resistance in the vector species of interest from that of human epidemiology (i.e., *f*), preventing hence β to drop too far from its initial β_0 value. As a result, introduced resistance transgene satisfying $\alpha_0 < \beta_0$ will be more likely to invade the targeted species while parasite-transmission will progressively shift toward the co-occurring vector species. In other words, as soon as two vector species coexist, the population invasion by the resistance transgene is facilitated in the targeted species at the expense of a quasi-null effect on the density of infected humans.

Deviations Caused by Differences in Vector-Parasite Interactions

To this point, we have implicitly assumed that vector resistance and susceptible genotypes respectively display the same interactive issues with all parasite genotypes they encounter. However, this assumption would require direct experimental testing, as nothing ensures that all parasite genotypes interact in the exact same way with all wild-type vector genotypes. This concern is even reinforced by noting that, more often than not, several of the *Plasmodium* spp that infect humans (*P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*) coexist and that they can have both positive and negative effects on each other's distribution.[28,29] In addition, experimental data from a rodent malaria model, *P. chabaudi*, and the vector *An. stephensi*, have refuted the assumption under which all parasite genotypes would impose the same detriment in fitness on susceptible vectors.[30] In another experimental infection set-up, one strain of *An. gambiae* selected for complete resistance to *P. cynomolgi* displayed a variable degree in resistance to other related parasite species and strains.[31] Overall, this raises the possibility that *Anopheles-Plasmodium* interaction may obey 'vector genotype x parasite genotype' interactive rules. Therefore, it may be possible that some natural or engineered vector genotypes would better resist infection than others when facing particular parasite genotypes but at the cost to be more easily infected by other parasite genotypes. How would this modify the conclusions of our qualitative model?

As long as the heterogeneity in *Anopheles-Plasmodium* interactive outcomes (i.e., infection success or failure) strictly concerns the wild-type genotypes, it would only induce heterogeneity in the selective advantage conferred by the release-resistance genotypes. Therefore, this would not lead to dramatic changes in our previous conclusions except that the evaluation of the α ratio would be more delicate. Indeed, any infection failure of a wild-type mosquito would

nullify the fitness advantage of the released resistance phenotype. Therefore estimation of the average advantage would necessitate an estimate of the proportion of vector-parasite natural encounters that actually result in vector infection. The situation would be more complex if the released-resistance genotype also conferred varying degrees of susceptibility and resistance depending on the *Plasmodium* genotype encountered. In this case, the possibilities for vector resistance evolution (and associated parasite evolution) will depend on the symmetry or asymmetry of the 'vector genotype' x 'parasite genotype' matrix cosigning the infection successes and failures (see refs. 25, 32-34 for details). Therefore, no pertinent conclusion can easily be drawn without previous study on how natural and transgenic *Anopheles* genotypes interact with the *Plasmodium* diversity encountered in the population targeted for a transgenic release.

Nevertheless, let us imagine that the genotype x genotype matrix suggests the possible disappearance of *P. falciparum,* to the benefit of other *Plasmodium* species. Would this lead to a public health benefit? At first glance the answer seems to be 'yes' since *P. falciparum* is presently the most virulent species for humans. Coinfection by other *Plasmodium* species has, however, recurrently been shown to protect humans from severe *P. falciparum* malaria, and it is reciprocally suspected that *P. falciparum* coinfection may provide some protection from serious *P. vivax* pathologies.[28,29] This raises new concerns for the public health outcome following a local change in *Plasmodium* genetic diversity. Whether or not this concern is valid in real life will remain unresolved as long as so little attention is paid to two complementary research axes. The first one concerns the diagnosis of *P.malariae* and *P. ovale* infections: if these infections are rarely reported as defining severe pathologies, is it because of their effectively low virulence or because of a misidentification of the parasite species involved in severe malaria diseases. The second question that would merit more attention concerns the pathology comparisons in mono- versus pluri-specific infections. The increasing prevalence of *P. vivax* and *P. falciparum* coinfection in Thailand could serve as a very good basis for such an investigation.[28,29] Other epidemiological situations can further improve our knowledge regarding the potential impact of *Plasmodium* bio-diversity on public health issues. For instance, a replacement of *P. falciparum* by *P. malariae* was suspected to have taken place in some areas of Tanzania, with such replacement being attributed to the efficacy of vector control programs, and potentially the longer duration of human infection by *P. malariae*.[35] Therefore, contemporaneous longitudinal records of malaria pathology (if they exist) may serve as another starting point to directly address the clinical effect of a change in *Plasmodium* genetic diversity.

Deviations Induced When Moving from Population to Metapopulation Levels

In reality, each population is not evolving independently from others. Instead, neighboring populations are connected by migration events that tend to homogenize their genetic composition. This migration effect is counterbalanced by within-population demography promoting genetic drift that tends to make populations genetically diverge from one another, and to impoverish within-population genetic diversity (especially when population size is small). Moreover, population extinction biases this migration/drift balance toward either homogenization or divergence depending on the precise mode of recolonization.[36] The phrase 'metapopulation' takes conjointly into account the effects of migration, genetic drift and extinction/recolonization processes.

Sub-populations in a metapopulation may often differ in the environmental forces pertinent for the phenotype of interest. As vector resistance-to-*Plasmodium* infection is likely to be a selected phenotype, variation in the environmental-induced selection pressures that act upon this phenotype must be considered (i.e., the density of humans that carry *Plasmodium* gametocytes infectious for the vectors, the parasite detrimental effect on the fitness of susceptible vectors, the vector fitness advantage and cost of parasite resistance). As a first approximation, assuming that the resistance fitness advantage and the associated fitness cost are high enough, the evolution of resistance can be forecasted by ignoring drift effects and focusing on migration/selection balance. Several models have investigated this question in the context of resistance to pesticides

(see refs. 37-40 and references therein); i.e., a case where among-population-variation in selection pressures is under human control since they correspond to the presence and absence of pesticide treatments. In this context, two key factors affect the possibility of resistance evolution within the metapopulation.[37-40] The first one relates to the geographical distribution of the environmental-induced selection relative to the migration range of the evolving species. This scale is important because it defines the average correlation in the selection experienced by mating individuals and by parents and offspring. The higher these correlations, the easier it is for resistance to evolve in some parts of the metapopulation. The second key factor concerns $\alpha = c/s$ (including the potential but crucial variation in dominance among the cost and advantage that are experienced in heterozygotes, see ref. 40 for details). Assuming an absence of parasite evolution in response to that of the *Plasmodium*-resistance in vectors, these conclusions can be extended to the case of mosquito-resistance to *Plasmodium*. Here, the pertinent environmental variation concerns the risk of *Plasmodium*-infection that the vector species of interest faces. Therefore, even if this risk is not under human control, any factor that will affect the correlation of such a risk among mating individuals and/or among parents and offspring will jeopardize the evolution of *Plasmodium*-resistance within a vector metapopulation. Quantitative forecasting will require not only knowledge of the geographical distribution of *Plasmodium* gametocytes, but also that of *Plasmodium* infectiousness and virulence toward wild-type vectors (i.e., components affecting the intensity of resistance fitness advantage).

Recap of the Biological Data Required to Correctly Forecast Vector and Disease Evolution

Recall that no resistance evolution can ever occur following the release of laboratory mosquitoes if *Plasmodium* infection does not impair the fitness of susceptible vectors. Direct tests of this assumption that include estimates of parasite detrimental effects on vector fitness remain nevertheless rare, with the exception of the work of Hurd and her collaborators.[41-43] These authors reported drastic reductions (from 15% to 48%) in mosquito fecundity in laboratory-controlled infections of *An. stephensi* and *An. gambiae* by *P. yoelii nigeriensis*, and in Tanzanian natural infections of *An. gambiae* by *P. falciparum*. This detrimental effect on female fecundity was found to last over at least three consecutive gonotrophic cycles, and to affect both strongly and weakly parasitized females (harboring >75 oocysts or ≈ 4 oocysts). Moreover, the extent of the reduction in fecundity was similar in strongly and weakly parasitized females during the 2nd and 3rd gonotrophic cycles (≈ 20% to 25% reduction). This suggests that there is weak parasite-dose dependence in the fitness detriment imposed by *Plasmodium* infection on their vectors. This would in turn suggest that there is a weak β-diminishing return on both α and the long-term evolution of *Plasmodium*-resistance in vector populations, and hence a high potential for a transgenic release to actually reduce *Plasmodium* burden in humans.

It nevertheless remains pivotal to test whether such 'favorable' characteristics tend to be the general rule among all the local possibilities of *Plasmodium - Anopheles* genetic combinations. As far as we know, this issue has not yet received attention. However, its importance appears reinforced by the recurrent reports of highly aggregated distribution of parasites among naturally infected vectors.[44] Such aggregation can either result from a high heterogeneity in the infectiousness among local blood-meals, or from a local variation in vector susceptibility to *Plasmodium* infections. Interestingly both possibilities are indirectly supported by our current knowledge of malaria biology. On the one hand, it has been proposed that, in some localities, 20% of a local human infectious reservoir may be responsible for 80% of malaria transmission.[45] On the other hand, naturally occurring *Anopheles* resistance to *Plasmodium* infection has begun to be identified.[47-49] Both possibilities would have dramatic consequences on the α-to-β comparisons that regulate the long-term evolution of the released transgenic resistance within vector populations. Indeed, they will respectively reduce β, through a reduction in the *f* probability to face infection risk, and increase α by reducing the fitness advantage of the released-resistance since natural vectors may be as able to resist infection as the released ones.

Therefore, no pertinent forecast of the local evolution of a released *Plasmodium*-resistance phenotype will ever be possible without previously knowing how natural *Anopheles* genotypes actually interact with *Plasmodium* ones in the locality chosen for this future release.

The need for data that document vector-parasite interactions appears even more crucial when addressing the probability for a vector to take an infectious blood-meal (parameter *f*, see section "From Biology to Minimal Formalization Able to Forecast Resistance Evolution"). Gametocyte burdens greatly vary with region of study and with human history of exposure and tend to reflect the overall asexual parasite density:[50,51] infections in the younger and therefore less immune children tend to produce higher densities of gametocytes than adults.[52-54] However, although increasing gametocyte density tends to result in greater infectiousness to mosquitoes, high gametocyte densities do not guarantee high mosquito infection rates.[55-58] Indeed, cryptic infected humans, apparently bearing no or very few apparent gametocytes, are capable of infecting mosquitoes and may contribute to a very significant proportion of the human transmission reservoir.[57-59] Such variability in human-to-mosquito transmission suggest several sources of variation that must be identified and taken into account in explicit evaluation of models of the transgenic strategy. So far, two sources of variation have been identified and a third may be hypothesized.

- First, the periodicity and intensity of malaria transmission are known to conjointly affect the distribution frequencies in gametocyte carriage among human age classes. In endemic low transmission areas, parasite prevalence rates are similar across all age groups and gametocytes are similarly frequent in all age groups.[60] As the transmission intensity increases, both overall and gametocyte parasite prevalence rates decrease with human age, reflecting the acquisition of immunity.[52,53] By contrast, in regions where malaria is epidemic, gametocytes are found at high frequency in all age groups and remain largely absent during inter-epidemic periods. Interestingly, these characteristics are entirely congruent with the assumption that *Plasmodium* parasite may be able to modify its human-borne life-stages to adapt to variations in vector availability.
- Second, the duration of gametocyte positivity in an individual infection varies with age and disease severity.[61] Both these factors are likely to vary not only with transmission intensity, but also with the local parasite specific diversity, and importantly, the efficacy of local health networks. Once more, this suggests a nonnull capacity of *Plasmodium* parasites for finding adaptive answers to variations in transmission constraints.
- Third, we can speculate on the occurrence of geographical differences in the match-pattern of *Plasmodium* and *Anopheles* genotypes that allow vector infection. Interestingly, two experimental studies provide indirect support for this.[30,31]

Thus, with a minimum of knowledge on the local transmission intensity, crude estimates of *f* can be made. How these characteristics will be altered by a decrease in transmission is uncertain, but such data should be available from previous studies where transmission has been reduced by extrinsic methods (e.g., bednet studies). Determining local parasite-vector adaptation and/or local adaptive capacity of parasites to respond to epidemiological changes may prove more problematic but potentially more important.

The Complex Biological Reality of Malaria: How Does the Acquired Knowledge Relate to the Pivotal Parameters of Resistance Evolution?

The Specific Diversity in Malaria Vectors

The engineering of resistant transgenic mosquitoes tends to focus on *Anopheles gambiae*, one of the major malaria vectors in the Afro-tropical region where the virulent *P. falciparum* is mostly found. However, more than one hundred *Anopheles* species have been described as malaria vectors worldwide,[62] and, even in the Afro-tropical region, the local co-occurrence of diverse vector species of *P. falciparum* looks to be the rule (Fig. 3). Furthermore, the so-called *An. gambiae* is a species complex regrouping gene-pools that evolve in relative independence

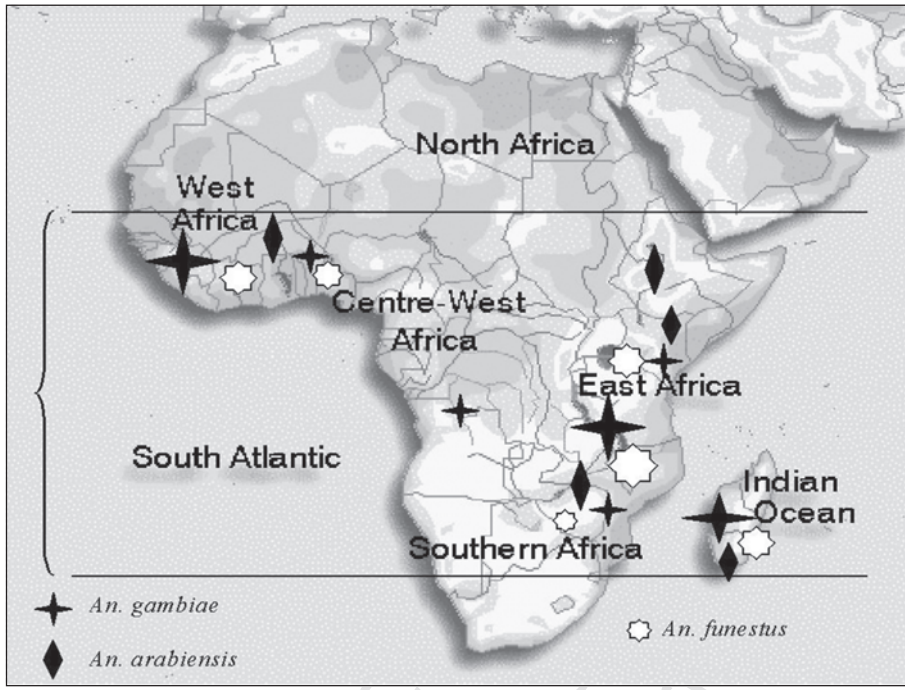

Figure 3. Biological diversity involved in malaria transmission: a neglected area of critical importance. More than one hundred *Anopheles* species have been described worldwide as vectors for malaria parasites.[62] Nevertheless, refractory transgenic mosquitoes mainly concern *An. gambiae* that is considered as the "main vector" in Afro-tropical region where *P. falciparum* is responsible for most malaria scourge. But: (i) *An. gambiae* constitutes a species complex including *An. gambiae s.s.*, *An. arabiensis*, *An. quadriannulatus*, *An. melas* and *An. merus.*[69] (ii) Other main vectors of *P. falciparum* frequently coexist with *An. gambiae s.s.* in this area. (See the figure below for a rough description restricted to "main" vectors). (iii) Other *Plasmodium* species (*P. vivax*, *P. malariae*, *P. ovale*) can also coexist with *P. falciparum* in Afro-tropical sites.[70] In the face of these observations, the major issue of transgenic release looks to focus on two interrelated questions: What could be the consequences of a release of refractory *An. gambiae s.s.* strains in Afro-tropical zone on the dynamics of the evolutionary relationships that *P. falciparum* entertains there with (i) its other vectors, and with (ii) the other malaria parasites?

from one another (Fig. 3). This raises different concerns regarding the potential of the transgenic release strategy. Firstly, there is no *a priori* guarantee concerning the biological compatibility between the engineered-strains to be released in a locality and the *P. falciparum* vectors living there. Secondly, the likely co-occurrence of competing vector species leads one to expect that the transgenic release strategy would at best modify the parasite-transmission-pattern with an uncertain impact on public health (see section "Deviations Caused by the Co-Ocurrence of Two Vector Species"). Circumventing these two difficulties by targeting several major vector species simultaneously would make the preparatory and production phases increasingly onerous, even when focusing on restricted geographical areas.

Such difficulty is exemplified by the joint investigation of malaria transmission and *Anopheles* diversity in the Senegalese villages of Dielmo and Ndiop, located 5km apart.[63,64] In Dielmo, the year-round presence of *An. funestus* guarantees continuity in malaria transmission. Six other *Anopheles* species were also identified, among which three were involved in malaria transmission to differing extents according to the season. Even more interestingly, *Anopheles* species that did not appear to be involved in malaria transmission in Dielmo were classified as good malaria

vectors elsewhere. As a result, the human-to-vector transmission pattern does not only involve distinct vector species within localities but these patterns are also heterogeneous along seasons and over African localities. This is not an exceptional situation as a comparable scenario was observed in Papua New Guinea, where anopheline diversity involves *An. koliensis*, three species of *An. punctulatus* complex, and six of *An. farauti* complex.[65] All these species are malaria vectors with both seasonal and geographical variation in their relative importance due to their variable degree of zoophily and ecology.[65,66]

The involvement of a vector species in malaria transmission actually defines the selection pressures acting on *Plasmodium*-resistance evolution. As a result, seasonal and geographical changes are likely to reduce the average correlation in the selection experienced among subsequent generations and among mating individuals that have previously migrated. This is worthy of note since such a reduction in correlation jeopardizes the evolution of resistance at the metapopulation scale (see section "Deviations Induced When Moving from Population to Metapopulation Levels"). This may be one of the reasons explaining why naturally arisen resistance to *Plasmodium* infections seems so rare in vector populations.

Wild Anopheles Vectors Have Naturally Developed Resistance to Plasmodium Infection

It has been argued that the lack of *Plasmodium* melanization by natural vectors to *Plasmodium* infection[67] indicates that the resistance fitness cost overcomes the resistance fitness advantage in nature.[68] However, melanization is not the only immune response of Insects, and alternative defense mechanisms have been shown to protect *Anopheles* natural genotypes from *Plasmodium* infections.[46-49] Given the lack of biological data in virtually all domains necessary for quantifying the fate of transgenic release (e.g., intensities of resistance fitness advantage and cost, distribution probability for the vector risk to encounter a virulent parasite, variation in the intensity of parasite virulence toward vectors, possibility that resistance to some parasite genotypes facilitates the transmission of others etc.), it would be of great help to start dissecting the evolutionary factors that actually regulate the evolution of such naturally occurring resistance mechanisms. Such investigations would certainly allow the incorporation of more realistic parameters in the explicit models aimed at evaluating the fate and epidemiological consequences of the release of laboratory-engineered resistant mosquitoes.

Conclusion on the Most Likely Results of the Release of Laboratory Resistant Anopheles

First of all, it is noteworthy that the majority of the present discussion does not strictly concern the particular case where mosquito resistance arises from trans-genesis. The only exception is the discussion on the possible use of genetic drive to reduce the high risk period of naturalization (see section "Trans-Gene Naturalization in Laboratory Experiments: The Actual Dimension of Inbreeding"). Otherwise, the entire discussion would be exactly the same whether the subject was transgenic resistant mosquitoes or selected pools of mosquitoes bearing naturally occurring resistant genes.

From this point, investigating the various sources able to affect the evolutionary fate of a released transgenic vector resistant to *Plasmodium* has led to a few qualitative conclusions. First, the major risks for trans-gene extinction are indeed expected to occur very soon after release (Fig. 1). Second, these major risks are more due to the laboratory-origin of the released mosquitoes than to the resistance characteristic it confers (Fig. 1). Therefore, even if some of these risks may be prevented before transgenic release (see section "Trans-Gene Naturalization in Laboratory Experiments: The Actual Dimension of Inbreeding"), the more likely outcome of a transgenic release strategy would be a rapid disappearance of the released trans-gene, inducing thus no visible change in malaria epidemiology, and hence neither public health improvement nor any medical and/or environmental risks. Third, possible consequences were pinpointed in the unlikely case where a released resistant genotype succeeds in persisting long

enough to actually modify the vector competence of the targeted species. Interestingly, the major expected outcome will rely on the specific diversity of the vectors, the parasites, or both (see sections "Deviations Caused by the Co-Ocurrence of Two Vector Species", "Deviations Caused by Differences in Vector-Parasite Interactions" and "Deviations Induced When Moving from Population to Metapopulation Levels"). Indeed, the likely outcomes of a persistent resistance trans-gene within a vector population would be a passive shift in the vector locally used by *Plasmodium* parasites and/or a modification in the *Plasmodium* genetic diversity that is locally circulating, either within or among *Plasmodium* species. Evaluating whether or not these outcomes would modify public health criteria would require data acquisition regarding eventual genetic variability of *Plasmodium* parasites in virulence-transmission tradeoffs.

Overall, it seems that the major predictable outcome of the transgenic release strategy would not concern public health but fundamental science! Indeed, a correct evaluation of the probability and consequences of the success of such a strategy will require reconsidering *Plasmodium* biology and genetics to its very roots, given the recurrent calls for the acquisition of remaining unknown data throughout the present discussion. For 50 years, malaria epidemiology has been based on the Macdonald's quantitative epidemiological model that ignores genetic variability among malaria parasites and their vectors. Since most of immediate consequences of transgenic release depend on parasite and vector genetic diversity, this classical model is no longer sufficient. On an optimistic note, as transgenic release strategy is so well suited for the media, we wager that the acquisition of the required but still missing data will soon benefit from the increasing attention. If this bet is correct, then the information pertinent to understanding the mechanics determining the dynamics of the malaria scourge will unquestionably increase.

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