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Evolutionary forces on *Anopheles*: what makes a malaria vector?

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In human malaria, transmission intensity is highly dependent on the vectorial capacity and competence of local mosquitoes. Most mosquitoes are dead ends for the parasite, and only limited ranges of *Anopheles* are able to transmit *Plasmodium* to humans. Research to understand the determinants of vectorial capacity and competence has greatly progressed in recent years; however, some aspects have been overlooked and the evolutionary pressures that affect them often neglected. Here, we review key factors of vectorial capacity and competence in *Anopheles*, with a particular focus on the most important malaria vector *Anopheles gambiae*. We aim to point out selection pressures exerted by *Plasmodium* on *Anopheles* to improve its own transmission and discuss how the parasite might shape the vector to its benefit.

Vectorial capacity and competence in malaria transmission

The vectorial capacity of a mosquito population largely determines the intensity of vector-borne disease transmission (Box 1). The vector competence (Glossary) is also a crucial parameter for the pathogen to be transmitted. In human malaria, vectorial systems are limited in number. Only *Anopheles* females are able to transmit *Plasmodium* to humans, and, among the more than 450 *Anopheles* species known, ~60 are considered to be actual vectors in the wild [1]. Vectorial capacity and competence also present quantitative features in the sense that some species have a major role in malaria transmission, and others have a minor role. Even at the species level, some populations or individual mosquitoes can have different impacts on transmission [1]. Research to understand the genetic determinants of capacity and competence has greatly benefited from the availability of the whole genome sequence for *Anopheles gambiae* [2] with the identification of candidate genes in progress. However, the different aspects of vectorial capacity and competence have not been uniformly studied, and some have been largely overlooked. For example, rapid progression has been recently made in mosquito immunity and olfaction genetics [3–5]; however, genetic determinants of parasite virulence and mosquito adaptation to human environments remain minor research areas. Moreover, evolutionary pressures on vectors, including the forces exerted by the parasites they transmit,

can have major consequences on malaria transmission and are rarely considered for their impact on malaria control measures. Here, we address the major aspects of vectorial capacity and competence and the evolutionary forces that affect them in *Anopheles*: the vector longevity; the duration of sporogonic development; the contact between the mosquito and vertebrate host suitable for the parasite; and the susceptibility/resistance of the vector to the parasite. We aim to point out selection pressures exerted by *Plasmodium* on malaria vectors to improve transmission, particularly in the *Plasmodium falciparum–An. gambiae* system, and discuss their relevance in malaria control.

Vector longevity

Malaria parasite development in vector mosquitoes requires passing through two epithelia and results in thousands of parasites [6]. The sporogonic development might therefore impose some degree of virulence (Glossary) and affect the fitness (Glossary) of the vector host. The infection-induced fitness cost can be expressed as a reduction of survival or fecundity [7] but an effect on survival would have a much higher impact on malaria transmission (Box 1) as the vector must live long enough to become infectious. Several mechanisms of *Plasmodium* virulence to mosquito vectors can be expected. Some were tested but mainly in experimental *Plasmodium–Anopheles* systems. The results on reduction of longevity are conflicting with many studies showing vector survival to be unaffected by infection, but some showing the opposite [8]. Potential mechanisms of virulence are: (i) the passage of parasites through the mosquito epithelia that can cause cell damage. It was observed that *Plasmodium* sp. causes

Glossary

Anthropophilic behavior: mosquito preference for breeding sites created by human activities, using human dwellings as resting places and humans as a source of bloodmeal.
Fitness: here, we consider it to be the success of individuals or populations in producing offspring, relative to others.
Refractoriness: intrinsic ability of Anopheline species or populations to not replicate and not transmit different *Plasmodium* species (or strains).
Resistance: mechanisms of host defense responsible for reducing parasite burden, it includes the immune response.
Susceptibility: intrinsic ability of Anopheline species or populations to replicate and transmit different *Plasmodium* species (or strains). It is approximated by experimental transmissions.
Tolerance: in tolerance, the host fights the disease by repairing damage caused by the infection, for example tissue repair.
Vector competence: most authors consider that it is synonymous to susceptibility.
Virulence: here, it is interpreted as the negative effects caused by the parasite on the mosquito in terms of survival or fecundity.

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Box 1. Vectorial capacity and basic reproductive rate

Vectorial capacity is defined as 'the average number of inoculations with a specified parasite, originating from one case of malaria in unit time that the population would distribute if all the vector females biting the case become infected'. It can be summarized by the Macdonald formula [61].

$$C : (ma^2 \, p^n / - \ln \, p)$$

m is the vector–host ratio (i.e. the anopheline density in relation to humans). **a** is the human feeding rate: the number of human bites per mosquito, per day. **p** is the daily survival rate (i.e. the probability of a mosquito surviving one whole day). **ln** **p** is the natural logarithm of **p**. The mosquito longevity is 1/p. **n** is the number of days required for sporogonic development (i.e. the time necessary for parasites to complete development from ingested gametocytes in the bloodmeal to sporozoites in the salivary glands).

   The basic reproductive rate (R_0) is the total number of malaria cases derived from one infective case that the mosquito population would distribute to man. R_0 must equal at least 1 for the disease to persist or spread. For values less than 1, the disease will regress. It is the product of the vectorial capacity, the infectiousness of vectors to humans (**b**) and humans to vectors (**c**), and the human infectious period (1/r):

$$R_0 = (ma^2 \, p^n / - \ln \, p) * bc * 1/r$$

c is closely linked to the vector competence of mosquitoes to *Plasmodium* species.

   In R_0, two parameters are more important than the others: '*a*' and '*p*'. '*a*', the human feeding rate is squared because the mosquito needs to bite twice to transmit the parasite, firstly to become infected, and secondly to infect, after completion of sporogonic development. For this reason, a small change in mosquito feeding preference will have a serious impact on malaria transmission. Even more importantly, '*p*', the survival rate, owing to its power *n*, has a disproportionate impact on transmission. As the sporogonic development of *Plasmodium* is relatively long (e.g. for *P. falciparum*: 12–23 days [30]) in relation to mosquito lifespan (2–4 weeks for *An. gambiae* [1]), mosquitoes can be infectious for a limited period only. Small changes in vector longevity can dramatically affect malaria transmission [62]. Modeling highlights that longevity is the factor with the biggest impact on the basic reproductive rate (R_0), even more so than initially suggested by Macdonald [61].

host cell damage, followed by apoptosis [9,10]; however, the effect of infection-induced apoptosis on mosquito survival remains unclear. Programmed cell death was observed in the midgut cells but also in the follicular epithelial cells in the ovaries, resulting in reduced egg production. The cell damage could therefore impact fecundity rather than survival [11]. (ii) The infection generates a mosquito immune response that can be costly. Parasite invasion of mosquito vectors in model and natural systems induces a massive immune response [12,13] and several studies in insects demonstrate that such an induced response can affect fecundity [14] or longevity [15]. In *Anopheles*, a reproductive cost was found [16] and *An. gambiae* mosquito strains selected for refractoriness to rodent parasites show reduced fitness compared with susceptible strains [17,18]. This suggests that developing an immune response induces a fitness cost, but more research is needed to establish whether there is an effect on mosquito survival. (iii) Competition for energy resources between the host and the developing parasites can have a negative impact on the host. In agreement with this hypothesis, a reduced survival was observed in *Anopheles stephensi* infected with rodent parasites and was most striking under the condition of glucose deprivation [19]; moreover, infection increases

mosquito sugar feeding [20]. (iv) Another potential cost of infection is that the parasite can affect mosquito behavior and cause mortality. In the natural *An. gambiae–P. falciparum* system, a higher feeding associated mortality was observed, probably as a result of a decreased efficiency in bloodmeal intake, and increased feeding activity [21]. This is, in fact, the only effect of infection on mosquito survival to be observed in this natural system. It could be the result of compensatory behavior by the mosquito to obtain sufficient blood for its trophogonic cycle, or a manipulation of the behavior of the mosquito by the parasite to increase its transmission by multiplying mosquito blood feeds [22]. Exploiting compensatory behavior can be considered a particularly well-developed strategy of manipulation by the parasite [23].

   Therefore, in spite of the potentially high virulence of *Plasmodium* to mosquito vectors, an effect on vector longevity is not always observed. The reduction of fecundity is a more common cost of infection and has only a minor effect on transmission. It is likely that evolutionary forces selected parasites affecting mosquito fecundity rather than that affecting mosquito survival, and this eventually benefited parasite transmission [11]. Moreover, meta-analysis of several published studies demonstrated that infection-induced mortality is less likely to be found in natural systems than in experimental vector–parasite species combinations [8], which could be explained by evolutionary forces acting between coevolved parasites and hosts. As both vector and parasite share the interest of vector survival, parasites might evolve towards low virulence, whereas, in parallel, mosquitoes less affected by infection would be selectively advantaged. For this to be true, virulence in *Plasmodium–Anopheles* interactions must have a genetic basis; this has been shown in some experiments, with both parasite and mosquito genetics affecting infection success [24–26]. However, the evolution of virulence is also highly dependent on environmental factors [19,24,25]. The fact that *P. falciparum* and *An. gambiae* share an evolutionary history, by contrast to laboratory model systems, might have led to the selection of mechanisms in both the parasite and the mosquito to reduce any negative impact on host survival and subsequently increase the chances of parasite transmission.

   Recent studies emphasize the concept of tolerance (Glossary) that might play a role in host defense against the parasite in parallel to resistance (Glossary). Tolerance is expected to be costly and could also impact survival or fecundity as the host spends resources to repair any damage the infection has caused. But, unlike resistance, tolerance to the effects of disease-induced mortality has a positive effect on parasite development [27,28] and could be an important mechanism in *Plasmodium–Anopheles* interactions. This opens a new avenue of research in the malaria vectorial system.

   Today, there is a crucial need to decipher parasite effects on mosquito fitness, the mechanisms involved and their genetic and environmental determinants to predict how parasite virulence evolves. This is particularly relevant for the prospect of using genetically modified mosquitoes (GMMs) to control malaria transmission. A recent study showed that the cost of genetic transformation

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Box 2. Vector longevity, insecticide resistance and malaria transmission control

Insecticides reduce mosquito longevity, the most important parameter of vectorial capacity. However, insecticide resistance limits the efficacy of vector–control measures and can interact with the parasite. New strategies could limit the emergence of resistance.

Selection pressures for insecticide resistance in malaria control
The use of insecticides for agriculture or public health results in a strong selection pressure [63]. In malaria vectors, multiple resistance mechanisms appeared independently and/or were able to spread in spite of strong gene flow barriers. For instance, several mutation events were selected independently in *An. gambiae* [64], and through introgression were passed to other members of the Gambiae complex [65].

Effect of insecticide resistance on infection cost
Genetic insecticide resistance was shown to impact the infection level in the invertebrate host. For instance, several studies showed that insecticide resistant *Culex* were more heavily infected by *Wolbachia* and suffered higher infection costs [66]. By contrast, in the case of filarial infection, insecticide resistant mosquitoes were less infected than susceptible ones. Insecticide resistance might affect parasite transmission in mosquitoes by changing potential redox reactions in several tissues and in doing so it was proposed that it could provide direct protection against infection [67]. To our knowledge, no studies have been published on the relationship between insecticide resistance in *Anopheles* and infection level/cost of *Plasmodium*, although they would be highly relevant to malaria control.

Killing mosquitoes responsible for malaria transmission and limiting insecticide resistance
Today, new elegant strategies are emerging to reduce vector longevity and subsequently reduce malaria transmission [68]. One concept is to use late-life acting (LLA) insecticides that would target older mosquitoes. The benefit of such a strategy is that it kills mosquitoes before they become infectious but after the majority of mosquito reproduction has taken place, limiting the selective pressure for resistance mechanisms. The LLA insecticides aim to kill the mosquito some time after exposure or target physiologically vulnerable mosquitoes based on the logic that older mosquitoes will be more sensitive. Insecticides with a new mode of action or new mode of administration are needed. Knowledge on mechanisms of senescence in mosquitoes or on the effects of pathogens could allow the development of alternative strategies such as the use of pathogens (e.g. *Wolbachia* or fungal pathogens [69]) with potentially synergistic effects with *Plasmodium* or insecticides on survival reduction.

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could be compensated for by the ability to resist infection in a laboratory model [29]. The potential success of the GMM strategy in the wild will be highly dependent on the balance between this cost and benefit to the mosquito, an area closely linked to the prevalence and virulence of infection. Moreover, vector longevity is likely to become a major target to control malaria transmission, for which we need a better understanding of the interactions between vectors, pathogens and insecticides in natural conditions (Box 2).

Incubation time of the parasite in the vector
The duration of sporogonic development is also a crucial factor in vectorial capacity. The earlier the sporozoites reach the salivary glands, the greater the opportunity to be transmitted; this therefore exerts a strong selective pressure to reduce the duration of sporogonic development. This assumption contradicts what we observe, as the development time in the vector is relatively long for *Plasmodium* (12–23 days [30]). Koella [22] hypothesized that this long duration of sporogonic development is necessary to produce a high number of sporozoites. Such a high number of parasites disturbs the efficacy of blood feeding and induces a higher biting rate and thus could be responsible for vector behavior manipulation [22]. In terms of transmission strategy, the parasite could be transmitted (i) as early as possible or (ii) later, when numbers of sporozoites are higher. As we observe this higher biting rate in infectious mosquitoes and a long sporogonic development time, it seems that the second strategy was selected. A higher biting rate comes with a greater risk of death and is likely to infer a manipulation cost on mosquito survival; this could, however, be compensated for by the benefit to the parasite in increasing its transmission.

A method to optimize the parasites adaptive interest in developing high numbers of sporozoites and shortening their development time is to carry out the cycle at its optimal temperature. It can be hypothesized that the parasite manipulates its vector in this way to increase transmission. Indeed, the sporogony dynamics are dependent on temperature; with a species-specific threshold, temperature is negatively correlated with development time [30]. A parasite-induced behavioral modification has been shown in *P. mexicanum*-infected phlebotomine sand flies, making them more attracted to higher temperatures than are non-infected individuals [31]. This suggests that *Plasmodium* might be capable of manipulating mosquito temperature preferences to increase their vectorial capacity, which warrants investigation in the natural human malaria system.

Human–mosquito contact and human biting rate
The density of vectors in contact with humans and the vertebrate host preference for mosquito bloodmeals are closely related. The anthropophilic behavior (Glossary) of *An. gambiae* is an important factor in its high vectorial capacity (Box 1). The hypothesis developed by Coluzzi explains the enhanced contact between humans and *An. gambiae* some thousands of years ago and the subsequent drastic changes in vectorial capacity [32]. An extensive penetration of forests began ~3000 years ago by Bantu populations, which have established agriculture through deforestation [33]. The *An. gambiae* ancestors, previously not able to survive in forests, could then find suitable sunny breeding sites and invade this new ecological niche. In parallel, a strong selection pressure against cattle, owing to trypanosomiasis, had the consequence that humans were the most frequent large vertebrate hosts available in such areas [32]. By providing the breeding sites and the bloodmeal to the newly arrived *Anopheles*, humans served as 'board and lodging', and selected the highly specialized species, *An. gambiae*, whose biology became very dependent on humans. This specialization to humans has been differentially selected in the members of the *An. gambiae* complex [32]. The adaptation of these different species to diverse environments and their related trophic behavior

was accompanied by the fixation of different chromosomal arrangements, which are known to protect coadapted alleles from recombination [34]. The association between chromosomal inversions and host preference provides evidence of a genetic basis for trophic behavior [35] and makes it susceptible to selective forces. The rapid adaptation of *An. gambiae* s.s. to humans and the specialization of the members of the complex to diverse environments is a clear illustration of their genetic diversity and plasticity.

The adaptation of *An. gambiae* ancestors to humans was accompanied by a dramatic increase in *P. falciparum* transmission. The traditional view of the story highlights the benefit of vector adaptation to its vertebrate host, but it could also be as a result of selection pressures exerted by the parasite to increase its transmission that might have strengthened the specialization. Several experiments revealed the ability of *Plasmodium* to modify the trophic behavior of the mosquito host. Vectors show a preference for biting gametocyte-infected human hosts [36] and pregnant women (who are generally more heavily infected) [37], and infected vectors are more aggressive [38,39]. One might think that the proportion of infected mosquitoes in nature would not allow strong selection pressure to be exerted by the parasite on mosquito behavior. However, considering the daily mortality of *An. gambiae* (estimated at 10–18% [40]) and the long sporogonic development (10–14 days), the 5% sporozoite infection rate frequently observed [41] means that a large proportion of *An. gambiae* are in contact with the parasite during their lifespan, and suggests that trophic behavior might be under selective pressures to increase parasite transmission [42].

Genetic determinants of adaptation to human environments and trophic behavior of malaria vectors remain almost unknown. Current investigation is based on the assumption that olfaction has a crucial role in behavior, at least in mosquito host choice for a particular bloodmeal. Recent descriptions of cellular and molecular olfactory components [4,5] open promising avenues for discovering how mosquitoes choose their vertebrate host for a bloodmeal and therefore the potential to modify its trophic behavior to limit malaria transmission.

Susceptibility and refractoriness to infection

A mosquito can be considered as a competent malaria vector if the *Plasmodium* parasite can internally complete its cycle from gametocytes in the bloodmeal to sporozoites in the saliva. We can distinguish different levels of susceptibility (Glossary) for human malaria parasites: a qualitative level with complete refractoriness (Glossary) of non-*Anopheles* species and a quantitative level among *Anopheles* species.

Numerous culicidae are in close contact with humans and can, in some cases, transmit infectious agents to them. Nevertheless, to our knowledge, no reports show human malaria parasite development in mosquitoes other than *Anopheles*. The density and the human biting rates of non-malaria vector mosquitoes can be high in areas of intense malaria transmission. Therefore, the ingestion of *P. falciparum* by non-vector species must be frequent; however, the vector competence has not been selected in a wide range of species. This suggests that crucial factors avoiding

infection in non-competent mosquito species must be highly differentiated from competent ones. The study of early parasite developmental steps in non-malaria vectors could therefore be used to point out essential mosquito factors in *Anopheles–Plasmodium* interactions and shed light on new targets for transmission blocking strategies.

In *Anopheles*, the susceptibility to human *Plasmodium* parasites appears to be more quantitative. Experimental infections of *Anopheles* species that do not naturally transmit *P. falciparum* often show low levels of infection but rarely none [43,44]. The outcome of infection depends on the mosquito response and the ability of the parasite to evade it. Indeed, the presence of infectious agents in the mosquito is considered as non-self and induces an immune response that can limit the infection efficiency [45]. Extensive work, mainly using model parasite–vector systems, partially deciphered the *An. gambiae* immune system [46,47]. Today, more effort is invested in research on natural vector–parasite systems. Genes playing a role in the mosquito response to *P. falciparum* have been identified [12,13]; a genetic island of resistance was mapped demonstrating that genetic variants play a role in susceptibility and resistance in mosquito vectors, and a candidate gene encoding a leucine-rich protein (APL1) shows allele-dependant resistance [48,49]. Comparisons of different vector–parasite combinations revealed that parts of the mosquito response mechanism are general and effective against several infectious agents [12,13], whereas others are more specific [13,50]. For instance, melanization appeared to be a baseline response to *Plasmodium* infection or other foreign bodies (e.g. sephadex beads) in *An. gambiae* [51] as in other mosquito species [43]. However, in the case of *An. gambiae* infected by *P. falciparum*, melanization does not appear as the major infection control mechanism, although it is occasionally observed in experimentally infected mosquitoes [48,52]. Also, silencing of key melanization genes did not affect the level of infection by *P. falciparum*: this contrasts with what was obtained when infected by *P. berghei* [50]. Thus, the mechanism of melanization appears to be a highly efficient mosquito response against non-self, except against the coadapted human malaria parasite. This suggests that *Plasmodium* either suppresses the immune response or develops evasion mechanisms [51,53]. The fact that key immune related mosquito genes are upregulated during *P. falciparum* infection [12,13,50] favors the hypothesis of evasion. Its capabilities to evade the response could be a major factor in vector competence.

The evolutionary arms race between parasites and hosts predicts constant evolution of mosquito refractoriness and parasite evasion mechanisms. The high diversity and plasticity of *Plasmodium* in vertebrate hosts leads to the assumption that the parasite evolves faster, and adapts more rapidly, than the host, and is expected to transmit more successfully through a local coevolved vector population, rather than a non-local one. In other words, the parasite would be the fastest runner in the host–parasite race. In agreement, several European *Anopheles* species that were tested for vector competence for *P. falciparum* showed the development of low numbers of parasites compared with *An. gambiae* [44]. These European species are

therefore competent with low efficiency for *P. falciparum* transmission. Thus, the high vector competence of *An. gambiae* could be partially because of the fact that African *P. falciparum* is well adapted to this vector, and the frequency and intensity of contacts between the parasite and the mosquito have determined the level of coadaptation. This would explain the efficient transmission of *P. falciparum* by its local vectors, but predicts that an introduction of *P. falciparum* in areas lacking this parasite, where vector populations are not coadapted, would not rapidly lead to a high level of transmission. However, it cannot be neglected that new contacts between *P. falciparum* and *Anopheles* are sometimes efficient: studies carried out in non-natural host–parasite combinations sometimes reveal high vector susceptibility [54,55]. It would be of great interest to be able to predict the success of new *Anopheles–Plasmodium* interactions in the context of drastic environmental changes such as current global warming.

The outcome of infection is parasite genotype–vector genotype dependant; no parasite has optimal transmission in all hosts and no host resists all parasites. As shown by Lambrechts *et al.* [56], *An. gambiae* isofemale lines can be highly susceptible to some parasite genotypes, and, at the same time, highly resistant to others. Furthermore, *Plasmodium* infectivity and mosquito susceptibility are environment-dependant [57]. Such genotype–genotype–environment interactions occur when one parasite genotype is more successful (better transmission) with one host genotype and less with another, but these interactions change when the host and parasite are exposed to different environments [58] and are known to affect insect immunity [59]. The mosquito genome has therefore been thoroughly searched for genetic determinants of vector susceptibility or refractoriness, although parasite genetics could be as important. Moreover, the efficiency of potential mosquito

resistance genes in natural situations might differ depending on environment and parasite genetics [60]. Studies on the effect of the environment and parasite diversity on the evolution of vector competence are crucial in understanding *Anopheles–Plasmodium* interactions and identifying suitable targets for malaria control.

Concluding remarks

Vectorial capacity and competence in the malaria system is the result of constant evolution of mosquitoes in relation to the other evolving members of the system (parasites and humans) and a changing environment (Figure 1). Here, we have outlined the role of the parasite on the evolution of vectorial capacity and competence. The parasite is likely to exert selection pressures on the vector to shape it to its benefit, such as it having a sufficient longevity, a specialization for feeding on humans and an immune system that it can evade. From this point of view, *P. falciparum* was extremely successful in selecting its main vector, *An. gambiae*. On other vectors, *P. falciparum* probably exerted lower selection pressures as a result of less frequent contacts between them, giving those vectors a less important role in malaria transmission. That is the case for the phylogenetically related species of the Gambiae complex to *An. gambiae s.s.*, a situation similar to that of most other species complexes. Studies on molecular evolution and the comparison of genomes between closely related species with differences in vectorial capacity and competence will shed light on selective forces specific to vectors. We expect strong evolutionary forces on genes involved in the most important parameters of vectorial capacity (longevity, host choice) and, maybe, lower selective pressure on others (vector immunity). Such studies could help to find new targets for malaria control. We currently face the great challenge of identifying genetic and environmental components of vectorial capacity and competence. Field studies
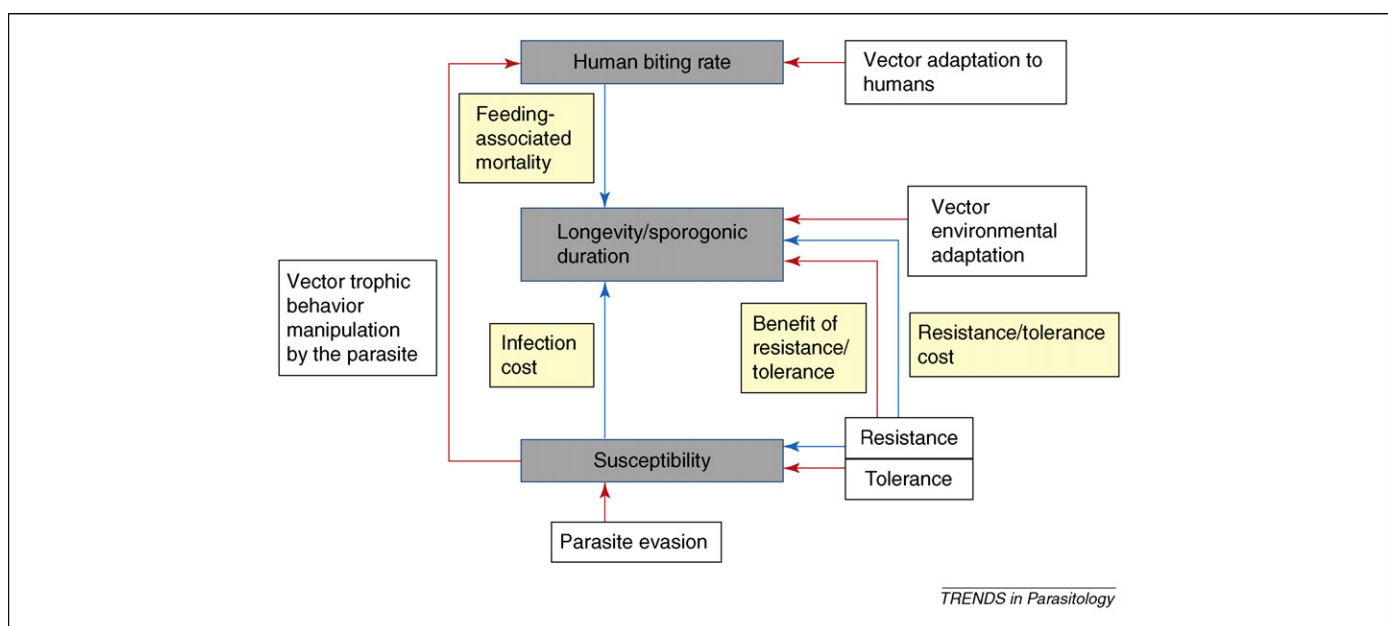
Figure 1. Schematic representation of feedback in vectorial capacity and competence. Red and blue arrows indicate positive and negative effects on malaria transmission, respectively. Key parameters of vectorial capacity and competence are shaded in gray. Human biting rate corresponds to '*a*', longevity to '*p*' and sporogonic duration to '*n*' in the vectorial capacity formula (Box 1). Susceptibility corresponds to vector competence (Glossary). Adaptive mechanisms are in white. They can induce fitness costs and benefits to the parasite or the vector (shaded in yellow) and have positive or negative impacts on transmission.

must be multiplied and take into account the evolutionary forces exerted in the wild, particularly by the parasite. Considering the evolutionary forces that affect vectorial capacity and competence is important in determining the epidemiology of malaria, and considering evolutionary ecology in the development of control strategies appears essential.

Acknowledgements

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