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The role of insecticides and malaria infection on the mosquito's behaviour and the consequences for malaria transmission

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Abstract

Insecticide treated bed nets (ITNs) are one of the most cost-effective techniques used for malaria control. While the repellent and irritant actions of ITNs offer personal protection, their insecticidal action contributes to community protection by lowering the number of infectious mosquitoes in the population. Despite this, the efficacy of ITNs to provide both personal and community protection may be affected by several aspects comprising sub-lethal exposure, malaria infection and insecticide resistance for example.

In this thesis, I tried to understand how insecticides and malaria infection may alter mosquito's behaviour in a way that may affect the efficacy of ITNs. First, I evaluate the role of sub-lethal exposures to insecticide that may follow irritancy on the mosquito's host-seeking behaviour and how malaria infection may interact with these exposures. Second, I investigated the ability of mosquitoes to directly bite through an ITN and evaluate whether that particular blood meal may reduce their fecundity and/or life-span. Third, whether resistance, spatial repellency and contact irritancy, are genetically correlated was evaluated using an evolutionary experiment. In the same experiment, the effect of insecticide resistance on the efficacy of ITNs to repel mosquitoes was investigated. In a fourth experiment, I tested whether malaria infection, especially sporozoite infection (the malaria stage that is infectious for human), by altering mosquito's behaviour, may affect the repellency of insecticides. Finally, I investigated the role of sporozoite load and apyrase activity (an enzyme that helps mosquitoes to locate blood) on mosquito's behaviours that are known to be subject to manipulation by the malaria parasites. These experiments showed how the evaluation of the efficacy of ITNs can become complex when considering that mosquitoes may be resistant to insecticides or malaria infected. The implications of the results of those experiments are discussed throughout this thesis.

Overall, this thesis highlighted that when evaluating the efficacy of ITNs, we may benefit by considering that both malaria infection and insecticide resistance may reduce the personal protection they offer. Thus, the results of this thesis will help to understand the real epidemiological impact of insecticide-based tools on malaria, particularly the impact of insecticide treated be nets.

Keywords

Malaria, *Anopheles* mosquitoes, Insecticide-treated bed net, Spatial repellency, Contact irritancy, Sub-lethal exposure, Insecticide resistance, Genetic correlations, Behavioural manipulation

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Résumé

Les moustiquaires imprégnées d'insecticides sont un des meilleurs moyens pour contrôler la malaria. L'action répulsive et irritante des moustiquaires imprégnées offre une protection personnelle aux utilisateurs, alors que leur action toxique, en diminuant le nombre de moustiques dans la population, augmente la protection communautaire. L'efficacité des moustiquaires imprégnées peut être affectée par les expositions sous-létales, les parasites de la malaria et la résistance aux insecticides.

Durant cette thèse, j'ai essayé de comprendre comment les insecticides et les parasites de la malaria modifient le comportement des moustiques et l'impact que cela pourrait avoir sur l'efficacité des moustiquaires imprégnées. Premièrement, j'ai évalué le rôle des expositions sous-létales aux insecticides sur le comportement de recherche d'hôtes des moustiques et j'ai testé comment les parasites de la malaria interagissent avec ces expositions sous-létales. Deuxièmement, j'ai examiné la capacité des moustiques à prendre un repas sanguin directement à travers une moustiquaire imprégnée ainsi que l'effet de ce repas sur la fécondité et la survie des moustiques. A l'aide d'une expérience évolutive, j'ai examiné si la résistance aux insecticides, la répulsion spatiale et l'irritabilité sont génétiquement corrélées chez les moustiques et j'ai testé les effets de la résistance sur l'efficacité des moustiquaires imprégnées. Quatrièmement, j'ai examiné si les parasites de la malaria, en particulier les sporozoïtes (le stade qui est infectieux pour l'homme), peuvent affecter l'action répulsive des insecticides. Finalement, j'ai examiné le rôle de l'intensité d'infection et l'activité de l'apyrase (une enzyme qui aide les moustiques à localiser le sang) sur différents comportements des moustiques qui sont sujet à être modifiés par les parasites de la malaria. Les résultats de ces cinq projets ainsi que leurs implications sont discutés en détail tout au long de cette thèse.

Globalement, cette thèse met en avant que lors de l'évaluation de l'efficacité des moustiquaires imprégnées, il serait bénéfique de considérer que l'infection par la malaria et la résistance aux insecticides peuvent réduire leur efficacité. Pour conclure, les résultats de cette thèse vont contribuer à mieux comprendre l'impact épidémiologique réel des moustiquaires imprégnées sur la malaria.

Mots-clés

Malaria, *Anopheles*, Moustiquaire imprégnée d'insecticide, Répulsion spatiale, Irritabilité, Exposition sous-létale, Résistance aux insecticides, Corrélation génétique, Manipulation du comportement

Content

Chapter 1: General introduction

1.1 Background

Vector borne diseases are the cause of more than 700'000 of human deaths per year [1] and are typically transmitted by arthropods that need blood resources for eggs production. For example, mosquitoes are vectors of a wide range of medically important diseases. They can transmit viruses as Dengue and Zika, filariasis or protozoans as malaria parasites [1,2]. These parasites are responsible for millions of deaths in the world with malaria as the main cause, e.g. 445'000 deaths in the world in 2016 with 91% of them occurring in Africa, with children younger than 5 years old being the most affected in the population [3]. Nowadays, malaria has a major socio-economic burden in Tropical Africa as it contributes to poverty and impacts the economic development of those countries [2–4].

Four main species of the genus *Plasmodium* cause malaria in humans: *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae,* and among these, *P. falciparum* is the most lethal [3]. However, last decades, a fifth species of *Plasmodium*, *P. knowlesi*, which is found in long-tailed and pig-tailed macaques located mainly in Asia, has been recognized as a new cause of human malaria [5]. The two most common species of human malaria are *P. falciparum* and *P. vivax*. Accounting for 99% of the malaria cases in 2016, *P. falciparum* is the most prevalent in sub-Saharan Africa while *P. vivax* is more prevalent outside of Africa [3]. Malaria parasites are transmitted by female mosquitoes of the genus *Anopheles.* While there is more than 400 *Anopheles* species worldwide, only about 10% of them are actually vectors of human malaria [2]. In sub-Saharan Africa, the main vectors are 2 species of the *Anopheles gambiae* complex, *A. gambiae sensus stricto* and *A. arabiensis* [2].

During last decades, intense work to control malaria has been done passing by an intense control of the mosquito vectors for example. The management of the mosquito's environment that help to reduce the number of potential oviposition sites in order to decrease mosquito's populations is a major component of integrated vector management (IVM) [6]. In addition, the use of chemical control methods with in particular the ones that help to reduce human-mosquito contacts, are particularly important as they directly affect malaria transmission. The two most common and cost-effective techniques that reduce contact between human and mosquitoes are the insecticide-treated bed nets (ITNs) and the indoor residual spraying (IRS) [7,8]. They both help to reduces malaria burden by repelling mosquitoes (preventing people from being infected) and by shortening or interrupting their life span [9]. The efficacy of ITNs to control malaria has been highlighted in several endemic areas with

several studies reporting a diminution of the number of malaria cases following intense distribution of ITNs [10–14]. Between 2010 and 2016, the proportion of people protected by ITNs increased drastically, with 50% of households having at least one ITN in 2010 while this number increased up to 80% in 2016 [3].

By repelling mosquitoes, ITNs offer valuable personal protection preventing mosquitoes from biting [15–18], and thus, transmitting their parasites. Further, by also killing mosquitoes, and thus reducing the number of infectious ones, they offer community-wide protection [13,19]. The efficacy of ITNs offering both personal and community protection is mainly attributed to the class of insecticides that is used to treat the net. Indeed, pyrethroids, which are the only class of insecticide recommended by the World Health Organization for the ITN impregnation [20], are known to spatially repel, irritate and strongly kill mosquitoes [21]. Therefore, more precisely, the pyrethroids repel mosquitoes in two steps; first some mosquitoes will be spatially repelled by the volatile molecules of the insecticide that are present in the air surrounding the ITN and thus will leave before having bitten the ITN users. Second, some mosquitoes that were not spatially repelled will be irritated by the toxic compounds of the insecticide by trying to pass through the net and will also leave before having bitten the users.

Although spatial repellency and contact irritancy both prevent mosquitoes from passing or biting through the net, contact irritancy, contrary to spatial repellency, implies that mosquitoes may contact the net and thus may be exposed to small (sub-lethal) doses of insecticide. Sub-lethal exposure to pyrethroid insecticides are known to have various effects on both insect's physiology and behaviour [22–24]. For mosquitoes, the effects may lead to an inhibition of their feeding behavior [25], may alter their egg production [25] or may reduce their life-span [26]. In addition, sub-lethal exposure to pyrethroids have been found to alter the mosquito's flying capacities, possibly impeding them in their host research [27]. Thus, because of their effects on mosquito's behavior and fitness, sub-lethal exposures (which may follow irritancy) may affect the efficacy of ITNs to give both valuable personal and community protection. In particular, the contact irritancy of ITNs, by altering mosquito's feeding capacities [25,27], may not only protect the ITNs users from subsequent biting tentative but also protect the non-users.

Most of the studies that evaluated the personal protection offered by ITNs tested whether mosquitoes are able to pass through holes in the net and whether they are able to take blood and survive to the exposure [28–31]. However, despite the presence of an ITN, mosquitoes may directly bite through the net when the users are touching this one. On the one hand, the exito-repellent action and the high induced-mortality of pyrethroid insecticides used to treat bed nets [21] should give no chance to mosquito to bite directly through an ITN and survive. On the other hand, it seems that only high

concentrations of insecticide prevent mosquitoes from directly biting through the net [32]. Indeed, Hossain and coworkers showed that only a concentration of 2.5 g/m2 of permethrin (a pyrethroids used to treat ITNs), which is 3 times higher than what is used to treat a commercially available permethrin-treated net (Olyset net, Sumitomo Chemicals), is able to completely prevent mosquitoes from biting [32]. They also reported that mosquitoes were able to survive at least 24 hours after the exposure to permethrin's doses up to 0.4 g/m2. Similarly, a second study showed that biting through a deltamethrin-treated net (another pyrethroid used to treat bed nets) resulted in mosquito's death when these where completely susceptible [26] but it was not reported how the treated net prevented mosquitoes from biting. Despite so, that mosquitoes may be able to bite through ITNs and survive may have strong implications for the epidemiology of mosquito-borne diseases, for it may reduce both the personal and community protection offered by the ITNs. Indeed, first, the fact that mosquitoes may be able to take blood directly through an ITN increases the risk of disease transmission. Second, that they may be able to survive to the exposure suggests that mosquitoes may become infected and further transmit their parasites.

Last years, the emergence of resistance to pyrethroids in mosquitoes threatens the efficacy of ITNs [33–35]. The evolution of pyrethroid's resistance is mainly attributed to the use of larvicide (for malaria control or by-product of agricultural use [36]). However, it might also be triggered by the repellent action of insecticides used to treat the bed nets [37]. Indeed, strong repellency would reduce the selective pressure for resistance as mosquitoes will be less killed by the insecticide, while, in contrast, weak repellency would increase the selective pressure for resistance [37–41]. The resistance to pyrethroids, by making mosquitoes less repelled by the insecticide, reduces the efficacy of ITNs to give effective personal protection. Indeed, for example, while a deltamethrin-treated net reduced the chance of mosquitoes to take blood by 72% when these were susceptible, it was only reduced by 43% when these were resistant [29]. In addition, other studies showed that a mutation in the voltage-gated sodium channel that induce nerve insensitivity, and which lead to knock-down resistance (kdr) [42] made *A. gambiae* mosquitoes less spatially repelled and less irritated by permethrin [43,44]. On the one hand, these results suggest that resistance decreases the personal protection ITNs offer, on the other, that reduction may be balanced by an increased community-wide protection. Indeed, by staying longer on the ITNs, resistant mosquitoes may receive a dose of insecticides that may kill them [29,44,45].

In addition to insecticide resistance, the efficacy of ITNs to protect their users might be threaten by the well-known behavioral manipulation of mosquitoes by malaria parasites. Indeed, the infection with the mosquito's infectious stages of malaria (sporozoites) increase by different ways the likelihood that mosquitoes enter in contact with a human host. First, sporozoite infection make mosquitoes more

attracted to human odors [46–48], although not all studies showed the same pattern [49]. Second, it increases the mosquito's probing time [50], biting rate [51] and persistence [52,53] and it makes them more likely to refeed after a partial or complete blood meal [54]. Finally, it increases the chance that mosquitoes bite several people [55]. By making mosquitoes globally more motivated to bite, sporozoite infection, through the reduction of either the spatial repellency or the contact irritancy or both, may increase the chance that mosquitoes bite the users, obviously reducing their personal protection at the stage where it is the most needed.

Nowadays, the mechanisms by which sporozoites make mosquitoes more motivated to bite are not clear. What we know is that sporozoites infection modify the saliva of mosquitoes in a way that decrease their blood feeding capacities. Indeed, by decreasing the activity of apyrase, an ADP-degrading enzyme that promotes easier and longer blood meals [56], sporozoite infection decreases the efficacy of mosquitoes to locate blood and as a result, their probing time is increased [50]. This suggests that during their limited time of contact with a person, infectious mosquitoes would have less time to take up blood, and therefore obtain a smaller blood-meals, than uninfected mosquitoes [54,55]. Thus, one possible mechanism, for at least one of the different alterations in mosquito's behaviour, is that sporozoites decrease either the quantity of apyrase, its activity or both.

Overall, to identify the real efficacy of ITNs to deliver personal protection, we should consider whether mosquitoes are able to directly bite through the ITN and whether the sub-lethal exposure generated by contact irritancy might affect mosquito's behaviour, especially their feeding capacities. Indeed, by achieving to blood feed through ITNs and surviving to it, mosquitoes may keep an active role in vector borne disease epidemiology. In contrast, by inhibiting mosquito's feeding behaviour, sub-lethal exposure may offer additional personal protection to ITNs users by reducing the chance that mosquitoes retry to bite or pass through the net and by increasing the protection of the non-users. In addition, to fully understand the epidemiological impact of insecticide-treated bed nets on malaria we should consider whether mosquitoes are resistant to pyrethroids and whether they are infected by malaria. Indeed, both may reduce the personal protection offered by the insecticide-treated bed nets. Finally, because of the potential impact of malaria infection on the efficacy of ITNs, it remains important to understand by which mechanisms the malaria parasites are able to affect mosquito's behaviour.

1.2 Thesis introduction

In this thesis I studied the impacts of insecticides and malaria infection on mosquito's behaviour. I tried to understand how alterations in mosquito's behaviour that are induced by both insecticides and

malaria infection might affect the efficacy of insecticide treated bed nets. To do so, I used two different species of mosquitoes that are both vectors of malaria parasites, *Anopheles gambiae s.s.* and *Anopheles arabiensis,* and two different malaria parasite species. One that cause malaria to rodents, *Plasmodium berghei*, and another that is responsible of hundred thousand human deaths each year, *Plasmodium falciparum*. The results of this thesis will hopefully enlarge our knowledge about the interactions between mosquitoes and their malaria parasites. They may help to understand the epidemiological impact of insecticide treated bed nets on mosquito-borne diseases like malaria. In particular, they may highlight that when evaluating the efficacy of ITNs to give effective personal protection, it is necessary to consider that mosquitoes may be affected differently if they are resistant to insecticides or infected with malaria parasites or both.

1.2.1 Effects of sub-lethal dose of insecticide on the host-seeking behaviour of mosquitoes and the potential impact of malaria infection

Insecticide treated bed nets (ITNs) offer personal protection by spatially repelling and irritating mosquitoes, thus by preventing the ITN's users from being bitten [15–18]. Contrary to spatial repellency, contact irritancy implies that mosquitoes contact the net and thus may be exposed to sub-lethal doses of insecticide which are known to inhibits their feeding behaviour [25] but also alter their flying capacities [27]. Thus, in addition to prevent the ITNs users from being bitten by mosquitoes, contact irritancy may not only increase the personal protection of the ITN by protecting the users from subsequent biting tentative but also increase the community-wide protection by protecting the non-users. Both aspects are useful when the mosquitoes are infected with malaria parasites, particularly sporozoites, the stage that is infectious for human. However, since sporozoite infection makes mosquitoes globally more motivated to blood feed [57–59], it is not worth to expect they might be differently affected by sub-lethal exposures that follow contact irritancy. In the second chapter of this thesis, I aimed to test, first, if short exposure to permethrin may affect the host-seeking behavior of *A. gambiae s.s.* mosquitoes, in particular, their motivation to bite. Second, I tested whether mosquitoes infected by either oocysts (the developmental stage in mosquitoes that is not infectious) or sporozoites of the rodent malaria parasite, *P. berghei*, were affected in the same manner than uninfected mosquitoes by the short exposure to permethrin. Third, because the dose of insecticides a mosquito might receive greatly depends on how they are irritated, I compared the contact irritancy of permethrin between infected and uninfected mosquitoes. The study may highlight how malaria infection could indirectly affect the control measures used to fight its vector, and thereby its own control. It will also provide new insights into malaria prevention by helping to understand the factors underlying the efficacy of our current techniques of control.

1.2.2 Can mosquitoes directly bite through insecticide treated bed nets? Consequences for their fitness

Classical procedures evaluating ITNs efficacy often overlook the particular case of mosquitoes trying to bite directly through the net. Indeed, because of the excito-repellent and insecticidal actions of the ITNs [7,14,28], it is generally assumed that it is barely possible for a mosquito to directly bite through the treated net and to survive to that exposure. However, a previous study showed that only concentration of permethrin that are 3 times higher than the ones used to treated actual ITNs (Olyset) are able to completely prevent mosquitoes from biting directly through the net [32]. Although, during this study, biting through treated net reduces the chance for mosquitoes to survive at least 24 hours, the results also suggested that a part of them survived. That mosquitoes might be able to bite through ITNs and might survive to the exposure may not only increase the risk of disease transmission during the blood meal through the ITN, but it also suggests that uninfected mosquitoes may become infected and may survive until they can transmit their parasites. However, surviving to a blood meal through an ITN may induced fitness costs to mosquitoes, for these latter may be exposed to sub-lethal dose of insecticide which may reduce their egg production [25] and their longevity [26]. Because of the impact it may have on mosquito-borne diseases epidemiology, in the third chapter, I aimed to test whether *A. gambiae s.s.* mosquitoes are able to bite through a widely used permethrin-treated net (Olyset plus®, Sumitomo Chemical) and the consequences it may have on their fitness, particularly on their fecundity and survival. I additionally evaluated how the insecticide, here permethrin, affects the blood feeding capacities of the mosquitoes by evaluating the time mosquitoes spend biting through the net and the blood quantity they ingested. Studying if mosquitoes are able to bite through insecticide treated bed nets and the costs it may imply is essential to fully understand the epidemiological impact of ITNs on mosquito-borne disease. In addition, it will provide new insights for the further development or evaluation of insecticide-based tools used to control vectors of medically important diseases.

1.2.3 Genetic correlations between resistance, contact irritancy and spatial repellency

Last decades the efficacy of insecticide treated bed nets has been threatened by the emergence of insecticide resistance, particularly the emergence of resistance to pyrethroids, the only class of insecticides used to treat the ITNs [33–35]. It has been reported that resistance particularly reduces the chance of the net to prevent mosquitoes from passing through it [29] certainly by reducing both the spatial repellency and contact irritancy [43,44]. The mechanisms by which mosquitoes resist to pyrethroids are generally divided in two groups, metabolic and target site resistance [42]. While the first one implies higher levels or activities of one or several proteins enrolled in the detoxification of the pyrethroid agents, the second one concerns mutations in target site that confer nerve insensitivity

to insecticides [42]. The most common mutation is a mutation in the voltage-gated sodium channel of mosquitoes that confers them a knock-down resistance (kdr) [60]. Because resistance seems to reduce both spatial repellency and contact irritancy [29,43,44], it is not worth to expect the genes of resistance to be genetically correlated with the gene responsible of the latter avoidance behaviours. In particular, the target mutation responsible of nerve insensitivity to insecticides might not only be selected through the selection of resistant mosquitoes but also through the selection of not irritated mosquitoes. Therefore, in the fourth chapter of this thesis, I aimed to evaluate whether the genes responsible of insecticide resistance in *A. gambiae s.s.* mosquitoes are genetically correlated with the one responsible of spatial repellency and contact irritancy. To do so, using deltamethrin, I tested the resistance, the spatial repellence and the contact irritancy of mosquitoes that I selected during 10 generations to be: 1) resistant, 2) strongly spatially repelled, 3) weakly spatially repelled, 4) strongly irritated or 5) weakly irritated. Investigated how resistance and avoidance behaviours in mosquitoes are correlated will help to understand how resistance might affect our current insecticide-based techniques of control. It will give new insights for the elaborations of new control tools that may interfere with the evolution of both type of resistance, the physiological and the behavioural ones.

1.2.4 How malaria infection in mosquitoes might affect insecticide's repellency?

To make insecticide-treated bed nets the most efficient against malaria, it would be ideal they mainly repel infectious mosquitoes, thus protecting users from being infected, and to weakly repel the other mosquitoes in order to maintain community protection by reducing the number of mosquitoes that may become infectious. However, because malaria infection is known to alter mosquito's behaviour in manner that makes them more motivated to have a blood meal [58,59], the predictions may be the opposites. Indeed, the general higher motivation of sporozoite-infected mosquitoes may have as a consequence that they may be less repelled by insecticides. In the fifth chapter of this thesis, I aimed to evaluate whether infectious mosquitoes are less repelled by insecticides compared to uninfected ones. To do so, I performed two studies in the laboratory with two different malaria systems and two ways of measuring repellency. In the first one I evaluated if *A. gambiae s.s.* mosquitoes infected or not with sporozoites of the rodent malaria *P. berghei* preferred to have a blood meal in the presence of an untreated net rather than a permethrin-treated one. In the second, I compared the mosquito's motivation to reach a blood source by passing through a deliberately holed permethrin-treated net (Olyset®, Sumitomo Chemical) between *A. arabiensis* mosquitoes infected with sporozoites of the human malaria, *P. falciparum*, and uninfected mosquitoes. Studying how malaria infection, especially sporozoite infection, might affect the efficacy of insecticides is critical as personal protection drastically depend on how infectious mosquitoes are repelled. In addition, it will help to understand how malaria

might affect its own control and thus will help to understand the factors underlying the efficacy of ITNs to control malaria.

1.2.5 Role of apyrase and sporozoite load on mosquito's host-seeking behaviours

The mechanisms by which sporozoites modify the behaviours of mosquitoes are not clear. What is known is that sporozoites when invading mosquito's salivary glands, in order to be transmitted with mosquito's saliva during the next blood meal, causes legion that modify the composition of the saliva impeding mosquitoes in their blood location [50]. In particular, sporozoites decrease the activity of apyrase [50], an ADP-degrading enzyme that helps mosquitoes to locate blood vessels and promotes easier and longer blood meals [56]. By decreasing the apyrase activity, sporozoites increase the probing time [50] and biting rate of mosquitoes [51], thus increasing the chance they bite multiple hosts [51], for both increased probing time and biting rate increase host-defense behaviors [61] and the probability of vector desistence [62]. If this idea is correct, the fact that *P. falciparum* sporozoite-infection intensity was positively correlated with the percent of multiple feeding of *Anopheles gambiae* [57] may imply that sporozoites load is negatively correlated with the apyrase activity. In the sixth chapter of this thesis, with *A. gambiae s.s.* mosquitoes infected with sporozoites of *P. berghei*, the main aim was to investigate the relation between sporozoite load, apyrase activity and mosquito's blood feeding behaviour. A second aim was to test whether the potential of mosquitoes to be manipulated by malaria parasites has a genetic basis. Indeed, because sporozoite-induced modifications in mosquito's behaviour reduce mosquito survival [63,64], it is not worth to expect natural selection to favour genotypes of mosquitoes that are able to avoid or decrease the manipulation. Understanding how malaria parasites alter the behaviour of mosquitoes might help to understand how these affect their own transmission and thus might give new insights for malaria control. In addition, understanding which factors are affecting the intensity of manipulation (e.g. mosquito's genotype) will help to understand how malaria parasites maintain an intense transmission in endemic area.

1.3 Experimental system

During this thesis, I used two mosquito species from the genus *Anopheles*, *A. gambiae s.s.* and *A. arabiensis,* and two malaria parasite species, the rodent parasite *Plasmodium berghei* and the human parasite *P. falciparum*.

1.3.1 *Anopheles* sp.

The genus *Anopheles* contains all the mosquito species that are able to transmit malaria parasites to human, but some species are also vectors of virus and filariasis [2]. From the *Anopheles* mosquitoes that transmit malaria to human, the two main species that are responsible of malaria in Sub-saharan Africa are *Anopheles gambiae s.s.* and *Anopheles arabiensis* [2]. While *A. gambiae* is highly anthropophilic, *A. arabiensis* take blood on several mammal species [65]. The *Anopheles* mosquitoes pass through a complex life cycle with four main stages: eggs, larvae, pupae and adults (Fig. 1). While the first three stages are aquatic, the adult stage is non-aquatic and it needs a blood meal to produce the eggs. The development period of *Anopheles* mosquitoes may vary in function of the environment but lasts between 7-20 days from eggs to adults [66]. During this thesis, the *A. gambiae s.s.* mosquitoes I used were coming from a Kisumu strain colony, which originated from western Kenya [67] and the *A. arabiensis* mosquitoes were coming from a colony established in 2006 in Sakamaganga (Kilombero, Tanzania) and maintained at the Bagamoyo Research and Training Unit (Tanzania).

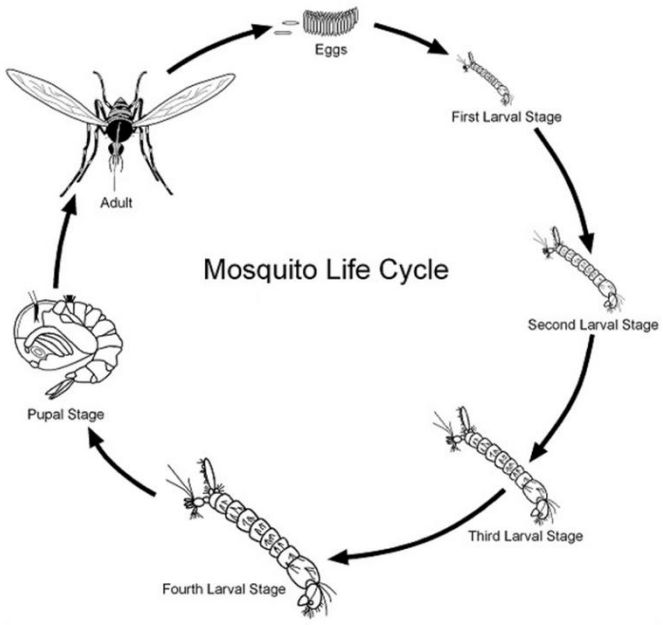

Figure 1 : Generalized life cycle of mosquitoes: Anopheles mosquitoes are holometabolous organisms and pass by four stages during their life cycle: Eggs, larvae, pupae and adults. The adult stage is the only non-aquatic stage and need a blood meal to lay eggs.

1.3.2 *Plasmodium* sp.

Malaria parasites from the genus *Plasmodium* are protozoans from the Apicomplexa groups. Although I used two different species of malaria parasite, *P. berghei* and *P.falciparum*, they follow the same life cycle in their respective hosts and vectors. During their life cycle in their hosts and mosquito vectors, the malaria parasites go through a complex development. A simplified description of the life cycle

within the mosquito (which is the part of the life-cycle that is important for this thesis) is shown in Figure 2. After taking up gametocytes (the infectious stage within a human), the parasite transforms first into an ookinete and then into an oocyst, which is the developmental stage of the parasite. Within the oocyst, the parasite replicates but cannot be transmitted. After a developmental period that may vary in function of the malaria species (10-12 days for *P. falciparum* [68,69] and 13-21 days for *P. berghei* [70]), the oocyst breaks apart to release up to several thousand sporozoites, which migrate to the mosquito's salivary gland, from where they can be injected into a human when the mosquito bites. The oocysts are, thus, a stage of the life cycle that is dedicated to replication and cannot be transmitted, whereas the sporozoites are the parasite's infectious stage which is used for transmission from a mosquito to a human.

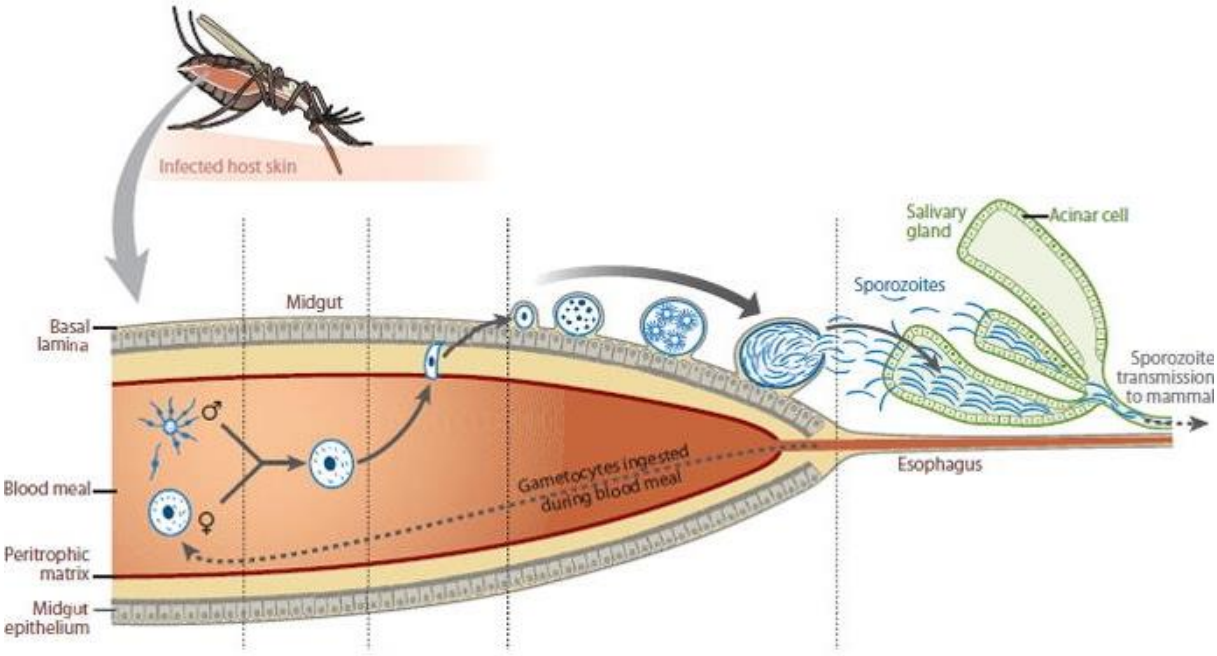

Figure 2: Life cycle of Plasmodium parasites within the mosquito vector. Modified from [68].

1.4 Research aims

The main goal of this thesis was to investigate the effects of insecticides, more precisely the one that are used to treated bed nets, and malaria infection on mosquito's behaviour. By using different insecticides, permethrin and deltamethrin, and different malaria system, human and rodent malaria, I tried to evaluate how it may impede the efficacy of insecticide treated bed nets to give both personal and community protection.

The thesis is composed of five main projects reported in the following chapter:

- **Chapter 2**

  Evaluation of the effects of sub-lethal exposure to insecticide that may follow irritancy on mosquito's host-seeking behaviour; the role of malaria infection in mosquitoes.
- **Chapter 3**

  Investigation if mosquitoes are able to bite through a commercially available insecticide treated bed net; the consequences of that blood meal for aspects of mosquito's feeding behaviour and fitness.
- **Chapter 4**

  The impact of pyrethroids resistance on both mosquito's avoidance behaviour, spatial repellency and contact irritancy; investigation for genetic correlation between the three traits.
- **Chapter 5**

  The role of sporozoite infection on the repellency of permethrin treated bed nets.
- **Chapter 6**

  Analysis of the interplay between sporozoite load and apyrase activity on mosquito's host-seeking behaviour; potential mechanism to explain malaria-induced modifications of mosquito's behaviour.

Chapter 2: The interaction between permethrin and malaria infection affects the host-seeking behavior of mosquitoes.

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2.1 Abstract

Insecticide treated bed nets (ITNs) help to control malaria by mechanically impeding the biting of mosquitoes, by repelling and irritating them and by killing them. We investigated a further possibility: that the brief, sub-lethal exposure to the insecticide experienced by irritated mosquitoes inhibits further blood-seeking by the mosquitoes. Since such inhibition would be particularly helpful in impeding the transmission of malaria if mosquitoes are infectious, we tested whether irritancy and inhibition differ between uninfected mosquitoes and mosquitoes carrying the non-transmissible stage (oocysts) or the infectious stage (sporozoites) of the malaria parasite *Plasmodium berghei*. In our laboratory setting, sub-lethal exposure to permethrin inhibited the host-seeking behavior of *Anopheles gambiae* mosquitoes by inhibiting their blood-seeking for almost 48 hours. Although infection by malaria did not affect the irritancy of the mosquitoes to permethrin at either the developmental stage or the infectious stage, both stages of infection shortened the duration of inhibition of blood-seeking. This suggests that the impact of ITNs will be weaker for malaria-infected than for uninfected mosquitoes. Our results will help to understand the impact of ITNs on the transmission of malaria and indirectly reflect the effectiveness of that vector control measure.

2.2 Introduction

In the last few decades, intensifying malaria control with insecticides that target adult mosquitoes, in particular insecticide-treated bed nets (ITNs), has decreased drastically the burden of malaria [7]. The efficacy of ITNs is partly due to their dual mode of protection: they protect the community by killing mosquitoes, thereby lowering the likelihood that mosquitoes live long enough to transmit malaria; and they protect individuals with the physical barrier of the net and the insecticide's repellency to mosquitoes, thereby reducing the risk that users are infected [37].

The repellency of insecticides has two components: spatial repellency and contact irritancy [71]. Spatial repellency occurs when mosquitoes detect the volatiles of the insecticide and then avoid the area near the ITN. It thus occurs before mosquitoes have had any physical contact with the ITN. Irritancy occurs when the physical contact of the mosquitoes with the insecticide makes them fly away. The relative importance of the two mechanisms of repellency depends on the insecticide. Permethrin, for example, is only slightly spatially repellent but strongly irritant, whereas DTT repels and irritates mosquitoes [71–73].

Since irritancy implies that mosquitoes contact the insecticide, and thus are exposed to a small (sub-lethal) dose of the insecticide, one of the differences between irritancy and spatial repellency is that the former may further affect the mosquitoes' physiology or behavior [22–24].

Most studies of sub-lethal effects on mosquitoes deal with larval exposure to pesticides and its effects on life-history traits (reproductive success, adult longevity, sex ratio) [74–78]) and on vector competence for, e.g. arboviruses [79–81] and malaria [82]. While sub-lethal exposure of adults has been less well studied, a general feature of exposure to insecticides, in particular pyrethroids, appears to be that it reduces the feeding rate for some time after exposure. This has been observed in tse-tse flies *(Glossina spp.*) [83,84], spider mites *Tetranichus urticae* [85] *Drosophila melanogaster* [86] and also in *Aedes aegypti* [25]. Sub-lethal exposure to pyrethroids also reduces the time of activation to flight and alters the flight direction of the mosquitoes *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles albimanus* [87]. These effects probably impede the subsequent search for hosts and may explain that the presence of ITNs reduces the number of mosquitoes that re-enter a house immediately after having exited it [88]. Overall, it thus appears that irritant insecticides can protect users of ITNs from being bitten and reduce the feeding rate of mosquitoes to protect non-users.

Both of these aspects of protection are most useful when mosquitoes carry sporozoites, since irritancy will then keep people from being infected. Unfortunately, since sporozoites make mosquitoes more motivated to bite [51–55], we might expect that they are less irritated by the insecticide. Indeed, we

found in a laboratory experiment that infectious mosquitoes (carrying sporozoites) were less repelled by ITNs [89].

We investigated with this study whether infection of the mosquito *Anopheles gambiae* by malaria at the non-infectious oocyst stage and the infectious sporozoite-stage affects the irritancy and the feeding inhibition induced by permethrin. We chose to work with permethrin because of its use on several commercially available ITNs (e.g. Olyset® and Olyset® Plus nets (Sumitomo Chemical Co. Ltd., Tokyo, Japan) [90]).

2.3 Methods

2.3.1 Ethics statement

All procedures that involved mice were conducted in accordance with the guidelines of the Swiss Tierschutzgesetz (Animal Rights Laws) and approved by the ethical committee of the University of Bern (Permit Number: BE109/13).

2.3.2 Mosquito and parasite

We used *Anopheles gambiae* mosquitoes from a Kisumu colony originating in Western Kenya [91], and a strain of the rodent malaria parasite *Plasmodium berghei* ANKA expressing green fluorescent protein (GFP). To infect the mosquitoes, we obtained mice infected with gametocytes from the laboratory of Professor Heussler from the University of Bern.

2.3.3 Mosquito rearing and infection

The tests for irritancy and for the sub-lethal effects on feeding motivation were done in two separated experiments. For irritancy, 600 mosquito larvae were reared individually in 12-well-plates in 3 ml of deionized water while for the sub-lethal effects experiment, for technical reasons, larvae were reared by 200 in trays (35x21x4cm) containing 1L of deionized water, for a total of 1200 larvae. In both experiments, larvae were reared with a standard level of fish food [91] (day of hatching: 0.04mg of Tetramin fish food per larva; one day 1 after hatching: 0.06 mg; age 2: 0.08 mg; age 3: 0.16 mg; age 4: 0.32 mg; age 5 and more: 0.6 mg). In each experiment, pupae were haphazardly moved to cages (21x21x21 cm) and the males were removed at most 24 hours after emergence to avoid that females were fertilized. The females were first maintained at 26±1 C° and 70±5 % humidity. One day before infection, we gradually decreased the temperature to 19±1 C°, which is the optimal temperature for *P.*

berghei development in the mosquito [70]. The mosquitoes were given access to a 6% sugar solution throughout the experiment except for the day before the blood-meal.

Even if the mice used to infect our mosquitoes were not the same between irritancy and sub-lethal effects experiments, we used the same procedure to infect our mosquitoes. Briefly, half of the 3-4 days old females blood-fed on gametocytic mice, the other half blood-fed on uninfected mice. We anesthetized mice with an intra-peritoneal injection of 8.5ml/kg of a mix of Xylazine Xylasol® (solution: 20mg/ml), Ketamine Ketasol ® (solution: 100mg/ml) and PBS [92]. We then placed the mice on the top of the cages and, let mosquitoes blood-feed for 20 minutes. One day after feeding, the females that were not completely engorged were removed from the cages. The mosquitoes were then maintained at 19 C° and 70 % humidity and with constant access to a 6% solution of sugar up to the tests.

2.3.4 Test for irritancy

Irritancy of the nets was measured for 40 infected and 39 uninfected mosquitoes 11 days after blood-feeding (when infected mosquitoes harbored oocysts) and for 72 infected and 64 uninfected mosquitoes 21 days after blood-feeding (when most of them harbored sporozoites). The mosquitoes were placed individually into the resting part of WHO test tubes [93]. After two minutes of acclimatization, we gently blew them into the exposure part of the tube where we had previously put a 0.75% permethrin-impregnated paper (WHO standard paper). We then recorded the time until their first jump (the time from landing on the paper up to flying in an attempt to escape from the paper [94]) and the number of times they jumped within two minutes. The mosquitoes were then frozen at -20°C until further analysis.

2.3.5 Test for motivation to blood-feed

We tested 72 infected and 80 uninfected mosquitoes 11 days after feeding (when infected mosquitoes harbored oocysts) and tested 96 infectious and 107 uninfected mosquitoes 21 days after feeding (when they harbored sporozoites). A third of the mosquitoes in each group were exposed either to a control paper (PY control), to 0.75 % permethrin-impregnated paper for 1 minute or to the permethrin for 2 minutes. They were placed in groups of six into the resting tube of the WHO susceptibility test tubes. After two minutes, we opened the trap and blew them gently into the test tube. After their exposure, the mosquitoes were transferred individually into 120mL plastic cups and given the opportunity to feed on a piece of cotton soaked with a 6% sugar solution.

To measure the mosquitoes' motivation to blood feed, we placed one of our arms onto each mosquito's cup repeatedly (2, 9, 24, 30, 36 and 48 hours after exposure to the insecticide) until it

responded and tried to bite. We also recorded the mortality of mosquitoes during the trials. All mosquitoes were frozen after the trials and kept at -20 C° for further analysis.

2.3.6 Detection of *P. berghei* infection

2.3.6.1 Oocyst detection

To detect the oocysts of *P. berghei*, we dissected the 11-day old mosquitoes in 10 µL of a 0.9% NaCl solution and examined the midguts under a binocular with a fluorescent filter for the GFP. We recored infection as the presence or absence of oocysts.

2.3.6.2 Sporozoite detection

We detected the sporozoites with a PCR of the head and thorax of 21-day old mosquitoes as described by Thiévent and coworkers [89].

DNA was extracted with DNAzol (MRC inc., Cinncinati, Ohio). Head and thorax were smashed with a pellet in 200 µl of DNAzol and were incubated for 20 min at 55 C°. The solution was then centrifuged at 20'000 g for 10 min. 170 µl of the supernatant were transferred to a new fresh tube containing 3 µl of PolyAcryl Carrier (MRC inc., Cincinnati, Ohio) and 100 µl of 100% ethanol conserved at -20 C°. After a centrifugation at 15000 g during 8 min, ethanol was discarded. DNA was washed with 0.8 ml of 75% ethanol (-20 C°) and the tube was centrifuged 5 min at 15000 g. Ethanol was discarded and we dried the pellet of DNA in a Speedvac for 15 min at 45 C°. Finally, the DNA was eluted in 50 µl of deionised water.

PCR amplification was done with the T3000 thermocycler (Analytik Jena AG (formerly Biometra), Jena, Germany) with 3 µl of DNA and 1 µl of a 10 µM solution of each primer (forward (5'-ACGATGATATAGATCAAAT-3') and reverse (5'-TACCTAAGCTTCTTGCGTA-3')). Primers amplify a 111 bp sequence of the merozoite surface protein-1 (MSP-1) gene of *Plasmodium berghei* NK65 and ANKA. Amplification was done during 40 cycles with denaturation at 95 C°, annealing at 54 C° and extension at 72 C°, each for 45s. Infection was visually confirmed by the presence/absence of the correct band of DNA by gel electrophoresis with a 2% agarose gel containing 9 µL of RedSafeTM Nucleic Acid stain (iNtRON Biotechnology, Korea).

2.3.5 Body size

The body size of mosquitoes was measured as their wing length. Wings were placed onto a slide and a picture of it was taken. Then using ImageJ, we measure the length of each wing from the distal end of the alula to the tip of the wing (the end of the vein R3) without the fringes.

2.3.7 Statistical methods

We assessed the contact irritancy of permethrin by analyzing (i) the time it took mosquitoes to jump off of the insecticide-treated filter paper and (ii) the jumping rate, which is the number of jumps per the remaining time after their first jump. The time until first jump was analyzed with a survival analysis with a mixed effect Cox model (*coxme* library in R [95]). The jumping rate was analyzed with a linear mixed model with the log-transformed jumping rate as the response variable. Both analyses included the infection status (infected or not) and the time after infection (11 or 21 days) as nominal factors, wing length as a covariate and the mouse on which the mosquito had blood fed as a random factor.

Our measure of motivation to bite was the time it took for mosquitoes to start biting after being exposed to permethrin. Because of our design (e.g. the irregular sampling points of 2, 9, 12, 24, 30, 36, 48 hours after exposure), we used an interval count analysis instead of a more classical survival analysis (see [96]). Since our data followed neither an exponential nor a Weibull distribution, we approximated a cox-hazard survival analysis by analyzing the number of mosquitoes feeding at a given time as a function of the interval of time between two measurements [96]). We added the exposure (permethrin or control), infection status (infected or not), the time after infection (11 or 21 days) as nominal factors, and wing length as a covariate. To obtain the results shown below, we removed non-significant interactions (with p-values greater than 0.25). Significance of the effects were assessed using a type 3 anova (*car* library in R [97]).

All analyses and graphs were done with the software R (version 3.4.3) [98] with the Rstudio interface (version 1.1.419) [99]. Mixed models (LMM and GLMM) were done using the *lme 4* library in R [100].

2.4 Results

For the irritancy experiment, 69.6% of the mosquitoes that blood fed on gametocytic mice were infected while for the sub-lethal effects experiment, 84.5% of them were infected. The remaining mosquitoes that did not harbored parasites but fed on infected mice were removed from the analysis. In total, we thus analyzed the behavior of 510 mosquitoes, of which 88 harbored oocysts, 132 harbored sporozoites and 290 were uninfected.

2.4.1 Irritancy

Eleven days after infection we measured irritancy for 26 oocyst-infected and 39 uninfected mosquitoes; 21 days after infection we measured irritancy for 52 sporozoite-infected mosquitoes and 64 uninfected ones. On average, it took about 24.3 seconds for mosquitoes to jump off of the insecticide-treated paper (Fig. 1), and they jumped about 4.8 times per minutes. Wing length had no effect on either time up to the first jump (χ^2=0.37, df=1, p=0.54) or the jumping rate (χ^2=0.03, df=1, p=0.87). Neither the time up to the first jump (χ^2=1.73, df=1, p=0.19) nor the jumping rate (χ^2=1.08, df=1, p=0.3) differed significantly between infected and uninfected mosquitoes. Neither the time up to the first jump (χ^2=1.19, df=1, p=0.28) nor the jumping rate (LMM, χ^2=0.08, p=0.78) was linked to the age of the mosquitoes. Finally, the interaction between the infection status and the time after infection had no significant effect on the time up to the first jump (χ^2=0.01, df=1, p=0.93) or the jumping rate (χ^2=0.06, df=1, p=0.3).

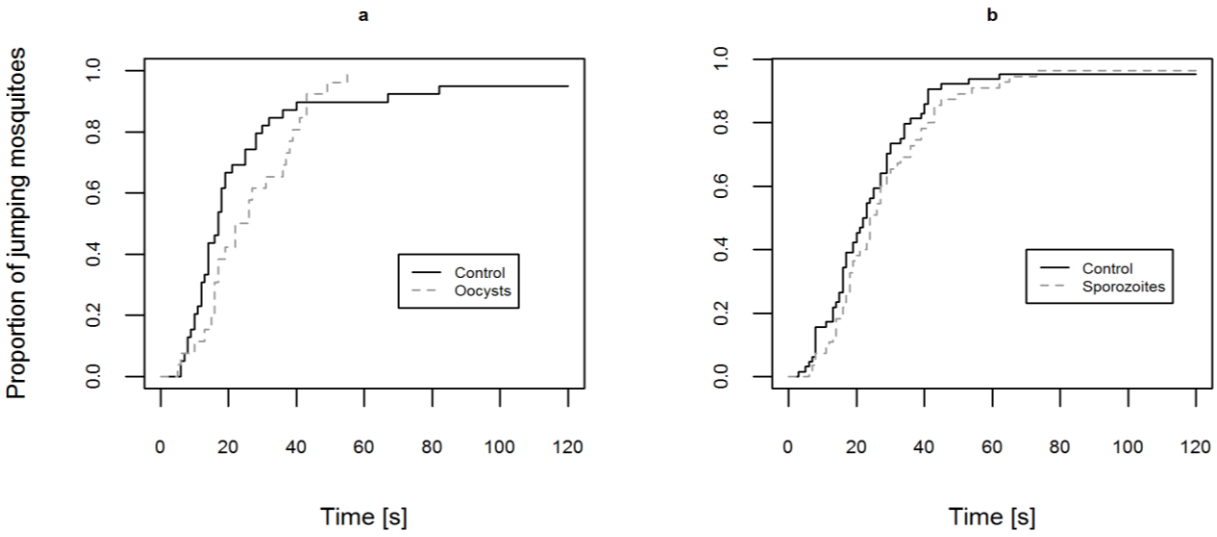

Figure1: Cumulative proportion of jumping mosquitoes in function of time. ***a:*** *Proportion measured 11 days after bloodfeeding, when infected mosquitoes were harbouring oocysts.* ***b:*** *Proportion measured 21 days after infection, when infected mosquitoes were carrying sporozoites in their salivary glands.*

2.4.2 Blood feeding motivation after exposition to permethrin

None of the mosquitoes died during the trials, i.e. within the 48 hours following the exposure to the insecticide-treated or untreated paper. We analyzed the motivation to feed of 329 mosquitoes. Of the 142 tested 11 days after infection, 62 were infected with oocysts and the remaining were uninfected. Of the 187 tested 21 days after infection, 80 were infected with sporozoites.

The summary of the statistical analysis of the rate of attempting to bite (called, for simplicity, biting rate from now on) as a function of time is shown in Table 1. First, the young mosquitoes (11 days after their bloodmeal) attempted to bite earlier than old mosquitoes (21 days after their first bloodmeal): 24h post exposition, 69.3% of the young mosquitoes had tried to bite while 61.7% of the old ones had.

Table 1 : Interval count analysis of the motivation to bite of mosquitoes after a previous permethrin or control exposure in function of time.

Factors	**X2**	**Df**	**P-value**
Infection status	0.97	1	0.32
Type of exposure	103.24	2	<0.001***
Age	0.01	1	0.92
Time	34.81	5	<0.001***
Infection status : Time	11.8	5	0.04*
Type of exposure : Age	9.1	2	0.01*
Type of exposure : Time	99.16	10	<0.001***
Age : Time	14.16	5	0.01*

Second, the biting rate decreased with time for mosquitoes that had been exposed to the control paper, suggesting that most of the mosquitoes that were willing to bite tried to do so at the first opportunity. When the mosquitoes had been exposed to the permethrin, however, the mosquitoes attempted to bite later and their biting rate increased with the time. Thus, permethrin significantly decreased the mosquito's motivation to feed for some time after exposure (Fig. 2). Indeed, 24 hours after the exposure for example, 88.5% of the mosquitos exposed to control paper had tried to bite while 57.5% of the mosquitoes exposed to permethrin for 1 minute and 50.5% of the mosquitoes exposed for 2 minutes exposure had tried to bite.

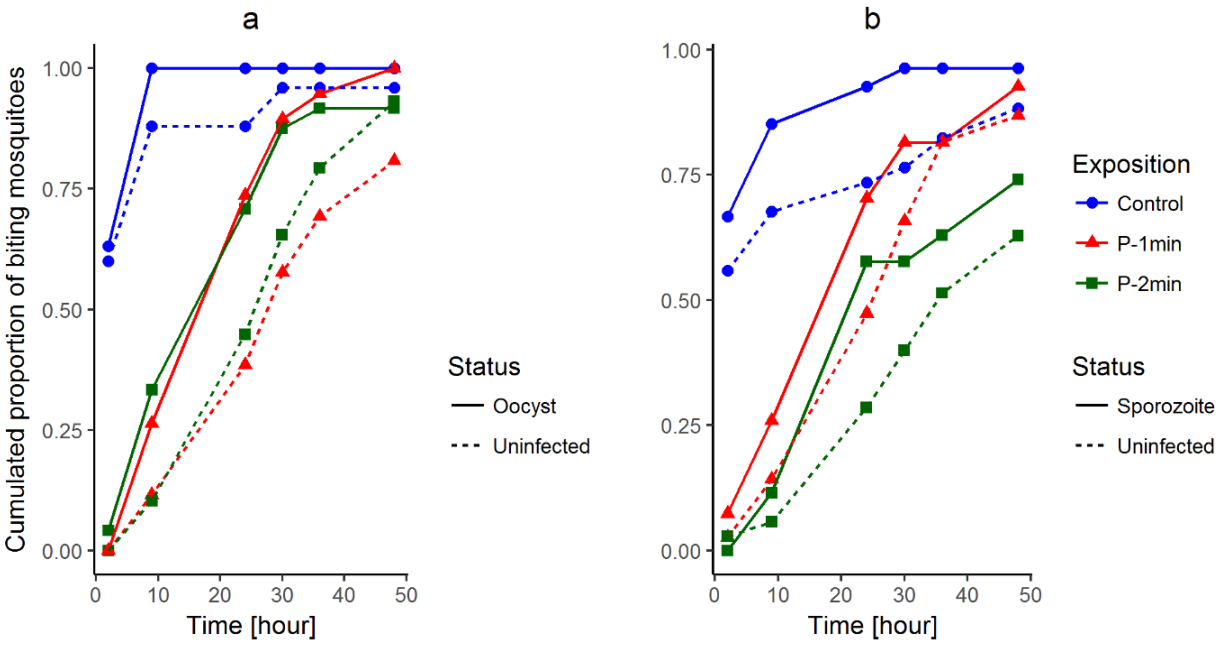

Figure 2 : Cumulated proportion of biting mosquitoes in function of time. Colors designated the type of exposure; Blue dots denote exposure on control paper, red triangles denote exposure to permethrin for 1 minute and green squares denote exposure for 2 minutes. Continuous lines represent the infected mosquitoes and the dashed lines represent the uninfected ones. ***a:*** *Cumulated proportion of biting mosquitoes tested 11 days after infection (so, infected with oocysts).* ***b:*** *Cumulated proportion of biting mosquitoes tested 21 days after infection (so, infected with sporozoites).*

Third, the effect of the exposure to permethrin differed between young and old mosquitoes. Thus, the time it takes for young mosquitoes to start to bite was similar whether they had been exposed to permethrin for 1 or for 2 minutes (Fig. 2a). In the older mosquitoes, however, the mosquitoes exposed to permethrin for one minute attempted to bite earlier than those exposed for two minutes (Fig. 2b).

Fourth, infected mosquitoes, independently of their age [i.e. independently of the stage of the parasite (interaction between age and infection status (not included in the final model), χ^2=0.2, df=1, p=0.65)] attempted to feed significantly earlier than uninfected mosquitoes. For example, 11 days after infection 81.5% of the oocyst-infected mosquitoes had tried to bite 24h after being exposed to permethrin, while only 57.1% of the uninfected ones had. The same pattern was found 21 days after infection with 73.6% of the sporozoite-infected mosquitoes that tried to bite 24h after exposure and only 49.8% of the uninfected ones.

2.5 Discussion

ITNs protect their users by creating a physical barrier between the users and the mosquitoes, by spatially repelling and by irritating mosquitoes. Here, we found that the irritancy of permethrin to mosquitoes is not affected if they are or not infected by malaria. We also found that irritancy has an

additional impact: short exposure to permethrin made mosquitoes less likely to try to bite for almost 48h without inducing any mortality. This inhibition was less long for malaria-infected mosquitoes than for uninfected ones.

The intensity or duration of sub-lethal exposure for mosquitoes depends mainly on how strongly they are irritated. Here we found that the irritancy (approximated in two different ways: time until first jump and jumping rate [94]) was similar for malaria-infected and for uninfected mosquitoes. Furthermore, the stage of the parasite with which mosquitoes were infected did not affect the mosquito's behavior. Our results thus suggest that when facing an ITN, mosquitoes, infected or not, would receive the same sub-lethal dose of insecticide. However, even though we found that most mosquitoes jumped at least once in the first minute of exposure, our design did not allow us to measure the persistence of mosquitoes that try to bite through an ITN. Therefore, it is worth mentioning that mosquitoes may be exposed for more than 1 minute when trying to reach a human host to blood feed.

Sub-lethal exposures to insecticides are known to affect the life history traits of insects in various ways [22,23] with, in particular, pyrethroids affecting their feeding capacities. Here we corroborated these results by showing that a short exposure to permethrin (which induced no mortality) alters the mosquito's host-seeking behavior. Indeed, the feeding motivation of mosquitoes exposed to permethrin for 1 or 2 minutes was inhibited for almost 48h. In addition, we found that the inhibition was stronger for old mosquitoes than for young ones, and that the inhibition was decreased by malaria-infection.

We had expected that infectious mosquitoes (so, those with sporozoites) would be less inhibited than uninfected ones, for the infectious mosquitoes are more motivated to bite [51–55]. Therefore, they should start to probe sooner after exposure than uninfected mosquitoes. Although this idea could explain the results found for sporozoite-infected mosquitoes, the fact that oocyst-infected mosquitoes were also less inhibited than uninfected ones does not support the idea, for the manipulation by oocysts goes in the opposite direction: they want to preserve mosquitoes from host-defense-associated-mortality, and therefore decrease their motivation to bite [54].

An alternative explanation for the weaker effect of permethrin on infected mosquitoes may be linked to oxidative stress. Indeed, the production of reactive oxygen species (ROS) is increased by both infection by malaria [101–104] and pyrethroids exposure [105,106]. After an infected blood meal, mosquitoes modulate this ROS production by expressing a higher level of antioxidant enzymes in the fat body [101]. Similarly, survival to pyrethroid exposure requires sufficient activity of antioxidant enzymes to avoid lipid peroxidation and cell damages [107]. It is therefore possible that the higher

antioxidant defense triggered by an infection with *Plasmodium* may, to some extent, help to deal with a further exposure to a pyrethroid insecticide. Furthermore, detoxification mechanisms may also play a role. It is known for example that following infection by *Plasmodium*, the cytochrome P450 CYP6M2, an enzyme involved in pyrethroid's detoxification [108,109], is upregulated [110]. Thus, because of the higher production of both antioxidant and cytochrome P450 enzymes, we expect infected mosquitoes to recover faster after permethrin exposure. This could help to explain why, in our study, they started to probe sooner after exposure. Such a link would have epidemiological implications, for it implies that infected mosquitoes would be more tolerant to pyrethroids. Glunt and coworkers [111] investigated this link – although with the opposite hypothesis, and reported no effect of *Plasmodium yoelii* sporozoite-infection on *Anopheles stephensi* mosquito's resistance to permethrin but reported a slight decrease in knockdown susceptibility for mosquitoes having had an infectious blood meal, compared to those fed with uninfected blood. However, as they mentioned, they did not separate mosquitoes that really harbored the parasite from the mosquitoes that fed on infectious blood but that were not infected, thus, this may have hidden any greater effect of malaria infection on permethrin susceptibility.

A further look into the different mechanisms implied in pyrethroid metabolism may also give an explanation to the observed longer inhibition of blood feeding behavior in older mosquitoes. Indeed, resistance to insecticides is often found to decrease with age (e.g. [91,112,113]). This may be explained by the age-related decline in P450 mono-oxygenases [114], esterases [115] and glutathione-S-tranferases (GST) [112] activity, the three main enzymatic systems responsible for pyrethroid resistance in mosquitoes [42,116]. Old mosquitoes may thus need more time to detoxify and recover after a pyrethroid exposure. It is therefore not surprising that in our study older mosquitoes responded differently to the dose of permethrin they were exposed to (1 or 2 min), while young mosquitoes showed similar inhibition response irrespective of the dose.

Whatever the mechanisms, the additional inhibition of mosquito's host-seeking behavior induced by permethrin may have strong implications for the epidemiology of malaria. Indeed, each time a mosquito is exposed to sub-lethal doses of permethrin following ITNs' irritancy for example, it will have to postpone its blood meal and therefore might lengthen gonotrophic cycle [44]. This might have strong implication for mosquito's fitness, but also for malaria transmission. Indeed, a reduction in the frequency of blood meals of uninfected mosquitoes could decrease the chance that they encounter malaria. Although our findings suggest that sporozoites decrease the inhibition of feeding behavior, the inhibition observed for the infectious mosquitoes might reduce the chances that the sporozoites are transmitted. In addition, that oocysts decrease inhibition may increase the chance that mosquitoes

infected with oocysts survive long enough to develop sporozoites and become infectious, thus, unfortunately, weakening the impact of ITNs.

ITNs offer personal protection by reducing the probability to get an infectious bite either by spatially repelling mosquitoes or either by irritating them. Here, we found irritancy to have an additional effect than just reducing the time mosquitoes pass on the net trying to bite or pass through it. Indeed, we found that sublethal exposure - 1 or 2 min – to permethrin, inhibited the host-seeking behavior of mosquitoes by impeding them to blood feed during almost 48 hours. We also found that malaria infection shortened the inhibition at both developmental (oocyst) and infectious (sporozoite) stages. Our results may help to get a better measure of the impact of ITNs for malaria control. On the one hand, the inhibition of the mosquito's host-seeking behavior might reduce the chance they encounter and/or transmit malaria. On other hand the weaker inhibition of infected mosquitoes helps the mosquitoes to survive the developmental period of the parasite.

Chapter 3: The ability of *Anopheles gambiae* mosquitoes to bite through a permethrin-treated net and the consequences for their fitness

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3.1 Abstract

Because of their efficacy to give valuable personal and community protection, insecticide-treated bed nets (ITNs) are one of the most effective protections against mosquito-borne diseases. Classical procedures evaluating ITNs efficacy often overlook the particular case of mosquitoes trying to bite directly through the net. Using a widely used permethrin-treated net (Olyset Plus®), we evaluated the ability of a susceptible strain of *Anopheles gambiae* to bite through an ITN and the following consequences for their fitness. We found that 71.4% of the tested mosquitoes succeed to take blood through the ITN, but that the time mosquitoes spent feeding, the quantity of blood they ingested as well as their subsequent fecundity and survival were all reduced by the ITN. Surprisingly, the mosquito's mortality 24h after the bloodmeal through the ITN was, however, much lower than predicted by time-response curve of the ITN. On one hand, these results suggest that when contacting the ITN, the users lose almost all the personal protection offered by the net, but on the other, the reduction in personal protection may be balanced by an increased community protection as the chance the mosquitoes survive enough time to transmit their parasite is decreased.

3.2 Introduction

Insecticide-treated bed nets (ITNs) are among the most cost-effective tools used to control malaria [7,117,118]. By reducing the number of malaria cases by 39 to 62% and child mortality by 14 to 29% [7], they help to save hundreds of thousands of people from dying of malaria every year [3]. The efficacy of ITNs results from several mechanisms of protection.

Bed nets protect people from being infected by malaria by creating a physical barrier between the user and mosquitoes. Mosquitoes can bite the user only if they find a hole through which they can penetrate the net or if they find a patch of skin that is touching the net and that they can bite through the net. Treating the net with an insecticide has several additional effects. First, ITNs can repel mosquitoes, so that they are less likely to approach the user. Second, they irritate mosquitoes, so that upon contact with an ITN some mosquitoes fly away rather than moving along the net to find a hole [28–30,44,119,120]. Third, if mosquitoes touch the net long enough (as they are trying to bite or when then they are resting on it after having bitten), the insecticide may kill them. By decreasing the number of infectious mosquitoes, this insecticidal effect offers community-wide protection [13,19] in addition to the personal protection.

The relative importance of the mechanisms of protection depends on the insecticide. Permethrin, for example, is only slightly spatially repellent but strongly irritant (reviewed in [71]). In one study in Tanzania, for example, treating a bed net with permethrin had almost no effect on the number of mosquitoes that entered experimental houses, but reduced the probability that a mosquito passed through a net by a factor of about 8 [30]. Nevertheless, the insecticide reduced blood-feeding success only by a factor or about 3 [30], which suggests that many mosquitoes bit through the ITNs without penetrating them. Indeed, although permethrin may keep mosquitoes from biting through the net, a laboratory experiment (with few mosquitoes) suggests that complete protection requires a higher concentration than what is used in commercially available ITNs like Olyset (1g/m^2) [32]; at 0.8 g/m^2 (slightly more than what was found on an Olyset net after 1 year of use [121]) more than half of the mosquitoes were able to bite through the net.

That mosquitoes can bite through insecticide-impregnated nets despite being irritated weakens the personal protection offered by the irritancy. It may, however, enhance community protection, for the mosquitoes may suffer from the contact with the insecticide while they were biting. In the study mentioned above, about a third of the mosquitoes that managed to bite through the net were knocked down and died [32]. This weak short-term effect of exposure to insecticides during biting may be complemented by long-term or sub-lethal effects. Thus, exposure to a Permanet 2.0 (a net treated

with 0.5% deltamethrin) reduces the survival rate of mosquitoes up to several days after exposure [26]. Mosquitoes irritated by insecticides are likely to stop their blood-seeking behavior for several days [25] (thus protecting the ITN-users and others from being bitten) and lay fewer eggs [25] (potentially reducing the number of mosquitoes in the population).

The aim of this study was to bring these ideas together and extend them by evaluating the ability of *A. gambiae* mosquitoes to bite through a new generation of ITN and its consequences for several aspects of the mosquitoes' feeding behavior and fitness. We used an ITN that is widely used in Africa: Olyset Plus®, which is treated with 2% (w/w) permethrin and 1% piperonyl butoxide (PBO), which slows down the metabolic degradation of pyrethroids [122,123]. In a blood-feeding assay, we measured the proportion of mosquitoes that were able to take blood through an untreated net and an Olyset Plus net, the time mosquitoes spent blood-feeding on the nets, and their blood-meal size. As fitness costs, we measured the mortality of the mosquitoes within 24 hours of their blood-meal and the fecundity and longevity of the surviving mosquitoes. In a separate experiment we measured the resistance level of unfed and freshly blood-fed mosquitoes exposed to the Olyset Plus net, so that we could test whether the bloodmeal itself affected the way mosquitoes survived to a contact with the ITN.

3.3 Methods

3.3.1 Mosquitoes

We used the insecticide-sensitive Kisumu strain of *Anopheles gambiae s.s.* mosquitoes originating from western Kenya [91]. We confirmed sensitivity of our colony by exposing 100 mosquitoes to 0.75% permethrin (WHO filter papers [93]) for one hour and finding that all mosquitoes died within 24 hours. During all the procedures, mosquitoes were kept in an insectary maintained at 26.5 ± 0.5 C° and 70 ± 5 % humidity with a 12:12 hours dark:light cycle.

3.3.2 Effect of ITN on blood-feeding behavior

We selected mosquito larvae haphazardly the day they hatched and reared them individually in 12-well-plates with the standard food regime of our lab: day of hatching, 0.04 mg TetraMin Baby® fish food per larvae; 1 day after hatching, 0.06 mg; day 2, 0.08 mg; day 3, 0.16 mg; day 4, 0.32 mg; day 5 and more, 0.6 mg. Pupae were moved to 21 x 21 x 21 cm plastic cages and adults were provided with a 6% sucrose solution.

Three to four days after emergence, we moved mosquitoes individually to 120 ml plastic cups covered with either a permethrin-treated net (Olyset Plus®) or an untreated net (Pharmavoyage® Trek) and let

them blood feed for 8 min on GH's arm. We measured the duration of their blood-meal as the difference between the time at which they started to probe and the time they pulled their stylet from the arm. Directly after the blood meal, we assessed the blood-meal size visually, removed unfed mosquitoes, moved blood fed mosquitoes to individual 30 ml plastic tubes covered with an untreated net and let them have access to a cotton ball soaked in a 6% solution of sucrose. Twenty-four hours after the blood meal, we recorded the number of dead mosquitoes. Three days after the blood meal, we moved the mosquitoes to 120mL individual cups that contained wet filter paper, and collected the eggs laid onto the filter paper the next day. To quantify hematin, we diluted the faeces that had been excreted in the 30mL tubes in 1ml 1% lithium carbonate (method described below). Every day, we assessed the survival of the mosquitoes.

3.3.3.1 Blood meal size

As previously described by Briegel and coworkers [126], we added 1 ml of 1% solution of lithium carbonate on the faeces contained in the 30 mL tubes and gently mixed the solution with a pipet until complete elution. The solutions were then transferred to 1.5 mL eppendorf tubes and kept at 4°C until assayed. We then vortexed the eppendorf tubes and transferred 200 µL of each solution to an ELISA plate along with a serial of standard dilutions of known haematin concentration, and we measured the absorbance at 387 nm with an ELISA plate reader. Each sample was measured in duplicate on 2 different plates. We calculated the haematin concentration from the standard curve specific to each plate and used the average concentration of the two replicates of each sample for the statistical analyses. Standards dilutions used haematin porcine (Sigma-Aldrich®, Saint-Louis, Missouri) with 8 different concentrations ranging from 0 to 50 ug of hematin per mL. Repeatability was 0.98 (calculated from samples replicates).

3.3.3.2 Body size

Wing length was used as a proxy for mosquito's body size. We placed the wings onto a slide, took a digital photograph of each wing and measured it with the software Image J from the distal end of the alula to the tip of the wing (the end of the vein R3) without the fringes.

3.3.3 Effect of bloodmeal on resistance

We reared larvae in groups of 200 in 35x15x5 cm trays containing 800 ml deionized water. This density limits competition among larvae [124]. We fed them with the standard food regime of our lab described above. We moved pupae to 21 x 21 x 21 cm plastic cages and provided adults with a 6%

sucrose solution. Four days after the first mosquitoes emerged, we blood-fed approximately 250 females for 8 minutes on Gaël Hauser's arm (GH). Directly after the blood meal, we measured the resistance of these mosquitoes and of approximatively 250 unfed females with the cone bioassay suggested by WHO [125]. We placed the mosquitoes in groups of 5 into a plastic cone (the upper 15 cm of a PET bottle of 8 cm diameter) fixed on a piece of an Olyset plus bednet. We exposed the mosquitoes for 1.5, 3, 5, 8, or 12 min and recorded mortality 24h after exposure. We replicated the exposure of 5 mosquitoes 10 times for each duration of exposure. To control for mortality induced by the manipulation itself, we also tested 10 replicates of 5 fed mosquitoes and 10 replicates of 5 unfed mosquitoes on an untreated net during 12 min. We did not find any dead mosquito after 24h in these control replicates.

3.3.4 Statistical analysis

For ANOVAs and LMs described below, the normality of model residuals was visually assessed and heteroskedasticity was checked with the *bptest* function (from *lmtest* library in R [129]). For Cox models, the assumption of proportional hazards was tested with the *cox.zph* function from the *survival* library. All analyses and graphs were done with the software R (version 3.4.4) [98] and with the Rstudio interface [28] (version 1.1.456).

3.3.4.1 Effect of ITN on blood-feeding behaviour

Blood-meal size

We described the blood-meal in four ways: a) the proportion of mosquitoes that tried to bite at least once (biting success) b) the proportion that succeeded to take blood during the 8 minutes they were allowed to (feeding success), c) the time required for the mosquitoes to start biting through the net (time to bite), d) the time they spent feeding until they detached or until the end of the allocated time (feeding time), and e) the quantity of haematin in the faeces, used as a proxy for the quantity of blood ingested (haematin level).

Biting and feeding success (binomial response variable) was analyzed with a Generalized Linear Model (GLM) with binomial error distribution. Time to bite was analyzed with a Cox proportional hazard regression model (from the package *survival* in R [128])), where the mosquitoes that did not tried to bite were censored. Similarly, feeding time was analyzed with a Cox proportional hazards regression model, where the mosquitoes that were still feeding at the end of the allocated eight minutes were censored. Finally, haematin level was analyzed with an ANOVA. Each model included the type of net (untreated or Olyset Plus) as an explanatory factor.

Fecundity and longevity

Fecundity was analyzed as: 1) the proportion of mosquitoes that laid at least one egg (*laying success*), and 2) the number of eggs laid by each female.

Laying success was analyzed with a GLM with binomial error distribution, where the type of net was included as explanatory factor and wing length was included as covariable. The number of eggs was analyzed with a multiple regression that included the type of net, wing length and haematin level as explanatory variables. Non-significant interactions were dropped from the final model.

Longevity was tested in two different ways: a) the proportion of mosquitos that survived the 24h following blood feeding (that is, the standard way of assessing the effect of insecticides on mortality), and b) longevity of mosquitoes that survived the first 24 hours (that is a delayed effect of the insecticide). Survival after 24h was analyzed with a Cox proportional hazards regression model. Both analyses included the type of net as an explanatory factor.

3.3.5.1 Effect of bloodmeal on resistance

We built time-response models with the *drm* function (*drc* library in R [127]). We used a 2 parameters log-logistic function, setting higher and lower limits for time-mortality curves to 1 and 0. Statistical comparison of the values of LT90 of each treatment was done with the *EDcomp* function of the *drc* package.

3.4 Results

3.4.1 Effect of ITN on blood-feeding behavior

3.4.1.1 Blood meal

101 mosquitoes were tested on an untreated bed net and 85 on an Olyset Plus net. Out of the 101 mosquitoes tested on an untreated net, the totality tried to bite (100% (95% CI: 95 to 100%), whereas on the Olyset Plus net, 75 out of the 85 tried (88% (95% CI: 79 to 94%) (X^2=16.34, df=1, p<0.001). Moreover, 100 out of the 101 mosquitoes tested on the untreated net succeeded to blood feed (99% (95% CI: 95 to 100%)), whereas only 60 out of 85 obtained a blood-meal through the Olyset Plus net (71% (95% CI: 60 to 80%)) (X^2=34.42, df=1, p<0.001). For mosquitoes that tried to bite, it took them an average of 35.0 seconds (± 12.9 (95% CI)) to start biting through the untreated net, but took them

about 50% more time (53.5 seconds (± 13.6)) to start biting through the Olyset Plus net (X^2=25.4, df=1, p<0.001).

More than 75% of the mosquitoes that bit through an untreated net were still feeding 400 sec after they had started to bite, whereas about 75% of the mosquitoes biting through an Olyset Plus net already stopped feeding after 175 sec and none of them fed more than 300 seconds (Fig. 1a; X^2=198.35, df=1, p<0.001).

Feeding time was also positively correlated to the amount of haematin excreted by the mosquitoes after blood digestion ($F_{1,136}$=652.2, p=0.001). Consequently, the amount of excreted haematin was 23% higher for mosquitoes that had fed through the untreated net (19.1 µg ± 1.9 (95% CI)) than for those that had bitten through the Olyset Plus net (15.5 µg ± 1.6) (Fig. 1b; $F_{1,136}$=6.8, p=0.01).

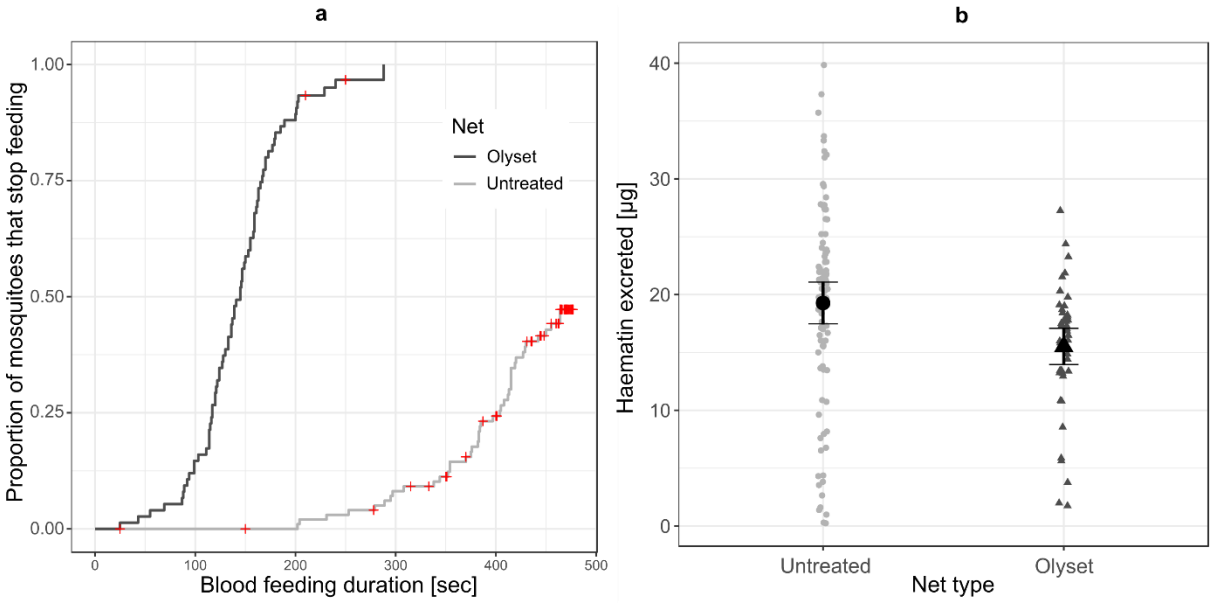

Figure 1. ***a*** *Cumulative proportion of mosquitoes with a given duration of blood-feeding (time betwen the beginning of probing and detaching). Red cross represents censored mosquitoes (the one that did not spontaneously detach and that were still biting at the end of the allocated time).* ***b*** *Quantity of haematin excreted by mosquitoes in function of the type of net they were able to feed through. Error bars show the 95% confidence intervals*

3.4.1.2 Fecundity and survival

The proportion of mosquitoes that laid eggs was 68% (95% CI: 58.4 to 77.1%) if they had fed through an untreated net and 77% (95% CI: 63.4 to 86.7%) if they had fed through an Olyset Plus net (X^2=1.7, df=1, p=0.19). Egg-laying success tended to increase with the mosquitoes' wing length (X^2=3.79, df=1, p=0.051). In contrast, among the females that laid eggs, the number of eggs laid per female (was greater if the mosquitoes had bit through an untreated net (129 ± 10 (95%CI)) than if they had bitten

through Olyset Plus (98 ± 14) (Fig. 2a; $F_{1,96}$=13.14, p<0.001). Fecundity increased with the mosquito's wing length ($F_{1,96}$=9.78, p=0.002).

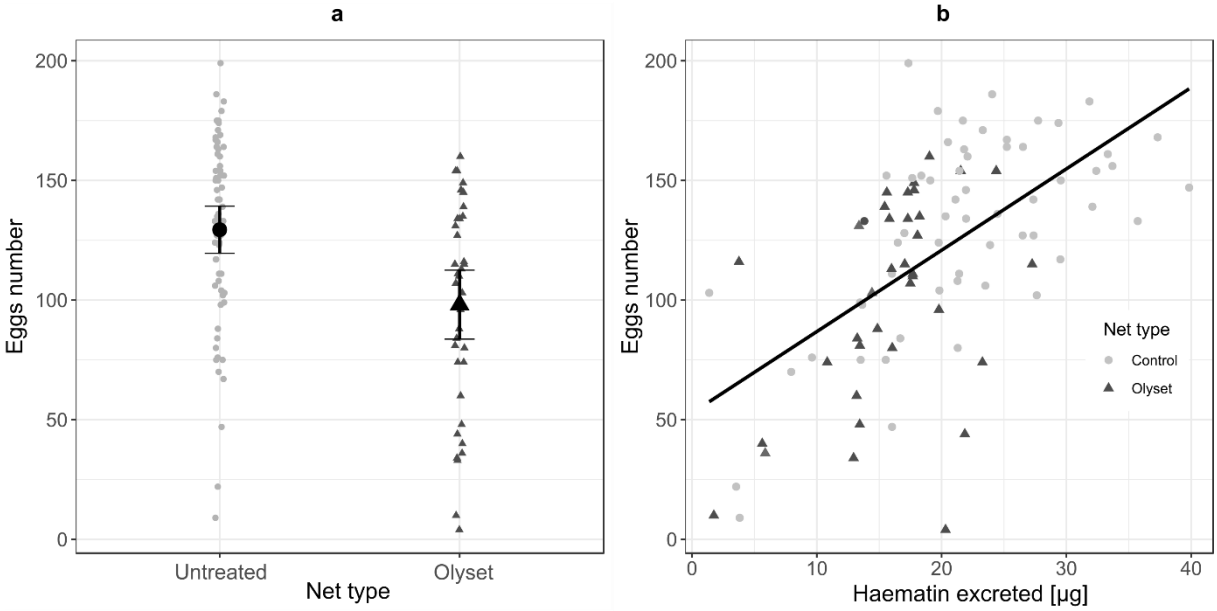

*Figure 2: **a** Quantity of eggs laid by mosquitoes in function of the type of net they bit through. Error bars show 95% confidence interval. **b** Correlation between the number of eggs laid by mosquitoes in function of the quantity of haematin they excreted. Black triangle represents the mosquitoes that fed through the Olyset plus net while the grey dot represents the ones that fed through the untreated net. The line represents the linear regression of the correlation.*

In a second model, haematin level was included in the multiple regression to look at possible mechanism responsible for the observed difference. In the latter, we found that haematin excretion level was positively correlated with eggs number (Fig. 2b; $F_{1,91}$=36.56, p<0.001), and that there were no longer any difference between the net types once haematin level was included in the model ($F_{1,91}$=2.44, p=0.12). The interaction between net type and haematin level was not significant ($F_{1,90}$=0.62, p=0.43) and was therefore removed from the model.

96 out of the 100 (96%) mosquitoes that had fed through an untreated net survived the 24 h after their blood meal, whereas only 51 out of 60 (85%) of the mosquitoes that had fed through an Olyset Plus did (X^2=5.87, df=1, p=0.015). Once they had survived the first 24 h, the mosquitoes on untreated nets lived another 8.0 days (±0.9 (95%CI)), and those that had fed through Olyset Plus lived 6.9 days (± 1.2 days) (Fig. 3; X^2=1.79, df=1, p=0.18).

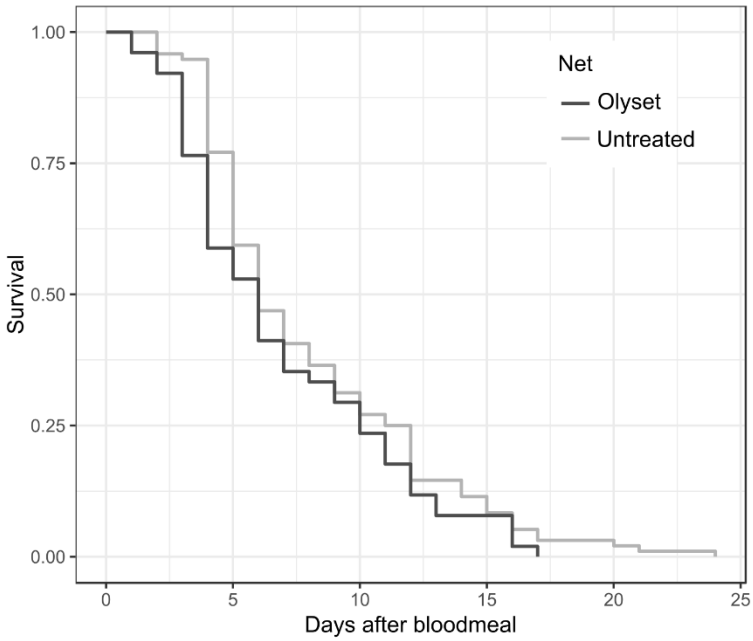

Figure 3: Mosquito's longevity in function of the type of net they could feed through. Day 0 was fixed at 24h following the blood meal. Black line represents the survival of mosquitoes that bit through the Olyset net while the grey one represents the mosquitoes that fed through the untreated net.

3.4.2 Effect of bloodmeal on resistance

251 fed and 287 unfed females were tested on an Olyset Plus net. The LT90 (so, duration of exposure that killed 90% of the mosquitoes within 24 hours) was 8'01'' for unfed females and 4'01'' for fed ones (t=-3.01, p=0.002, Fig. 4).

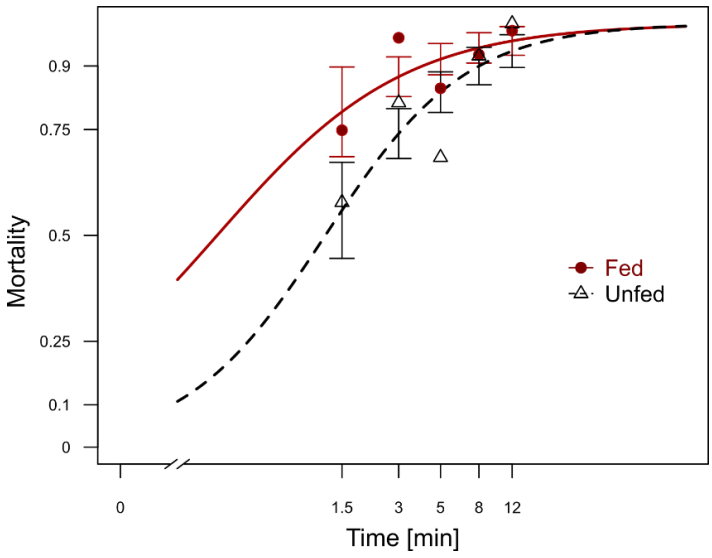

Figure 4: Time – mortality curve of unfed (black dashed line) and freshly blood- fed (red solid line) females exposed to an Olyset Plus net following the WHO cone bioassay procedure.

3.5 Discussion

Insecticide treated bed nets prevent mosquitoes from biting their user by irritating them. With this study, we confirm the permethrin of the Olyset plus net to be irritant for mosquitoes [43,44,71]. Despite that irritancy, the ITN did not completely prevent mosquitoes from biting or blood feeding through the net as 88% of them effectively tried to bite and 71% succeeded to take a blood meal. However, blood meal through the permethrin-treated net induced fitness costs for mosquitoes as it reduced both their chance of surviving and their fecundity, corroborating the results from previous study [25,26,32].

The irritancy of the Olyset plus net was confirmed by the fact that the mosquitoes took more time to start blood feeding and spent much less time feeding through the treated net compared to the untreated net. Although we could not distinguish the role of spatial repellency and contact irritancy for the delay in the time mosquitoes took to bite, the reduced time mosquitoes spent biting through the net confirms that permethrin is strongly irritant [43,44,71]. In addition, by reducing the duration of the blood meal, the irritancy of the Olyset Plus net decreased the quantity of blood mosquitoes were able to take and, as a consequence, the number of eggs they laid. We thus also confirmed that sub-lethal exposure to pyrethroids may alter the number of eggs mosquitoes are able to produce [25]. Our results however suggest that the mechanism involved in the reduction of fecundity after an ITN exposure is the reduction of blood meal size caused by a reduced feeding time. Overall, these results suggest that the ITN may not only prevent mosquitoes from biting by irritating these ones but may also reduce the density of mosquitoes, through a reduction of their fecundity.

Despite that the Olyset plus net strongly irritated the mosquitoes, our results showed that it prevents only 12% of the mosquitoes from trying to blood feed through the net and that 80% of the ones that tried to bite were able to take blood. Therefore, when contacting the net, the personal protection offered by the Olyset Plus net may be drastically reduced. This might have strong implications for the epidemiology of mosquito-borne diseases like malaria. Indeed, although the Olyset plus net reduces the biting duration, when the user contacts the net the transmission potential may only slightly be reduced, for *Plasmodium* sporozoites (the stage that is infectious for human) are mostly released at the beginning of the bite [130,131].

Because the WHO recommended the ITNs to kill 80% of the mosquitoes within 24h after being exposed for a duration of 3 min in cone assay [125], we first expected most of the mosquitoes to not survive after a blood meal through the Olyset Plus net. However, we found that long-term mortality was not affected by the ITN, and that there was only a difference with the untreated net at 24h after the blood

meal. This result shows that permethrin exposure, despite the presence of a synergist (PBO), may mostly have a short-term impact on survival. But this impact was surprisingly low (18% mortality at 24h). Indeed, according to our dose-response model, mosquitoes being exposed to an Olyset Plus net during 2 min 23 s – the average feeding time recorded– should have experienced 68% (unfed females) or 85% (fed females) mortality at 24h. A probable bias is that, contrarily to the mosquitoes used to build the dose-response model (cone bioassay) that were all exposed in a similar way, the mosquitoes used in the feeding experiment had the choice to bite (and being exposed) or remain in the bottom of the test tube. Thus, it might be that only the most resistant individuals succeeded to take a bloodmeal, which may explain why they also survived to the contact with the net. However, even if we consider that all the mosquitoes that did not bite would have bitten and have died in 24h, then the recorded mortality would have been 39% instead of 18%, which is still considerably lower than what was predicted by the dose-response model for a comparable time of exposure.

Another possibility to explain the low mortality induced by the blood meal through the treated net may come from the blood meal itself. Particularly, we hypothesize that when both the blood meal and permethrin exposure happen simultaneously, it helps mosquitoes to reduce the detrimental effect of permethrin. Indeed, the only presence of blood in the midgut cannot explain the low mortality experienced by the individuals that blood fed through the Olyset Plus net, for the fed mosquitoes were found to be more susceptible than unfed mosquitoes in the resistance assay. Therefore, we briefly introduce hereafter two other possible mechanisms that might be implied. First, it is known that both a blood meal [101] and pyrethroid exposure [106,105] induce an increase in reactive oxygen species (ROS) which is quickly followed by a higher expression of different antioxidants in the midgut and the fat body [101], as found in some pyrethroid resistant mosquito strains [133]. Thus, one first possibility may involve an oxidative based interplay between the blood meal and permethrin exposure, which may finally reduce the damages induced by the insecticide. However, why this mechanism was no longer active a few minutes after the blood meal, as we tested in the resistance assay, is not clear. A second possibility may involve temperature. Because during a blood meal, mosquitoes see their body temperature rapidly increasing [134] and because the toxicity of pyrethroids decreases while temperature increases (i.e negative temperature coefficient) [135–137], they might less suffer from being exposed to pyrethroids. This hypothesis is also more consistent with the results obtained via the cones-bioassay: fed mosquitoes did already cool down when they were put on the Olyset Plus net and were therefore not protected anymore by the high temperature reached during the blood meal.

The possibility for the blood meal to mitigate toxicity of pyrethroids may be of high importance when evaluating ITNs efficiency and their insecticidal properties, as the protection they offer might be overrated in that particular case. Indeed, in our study, although we confirmed the commercially

available permethrin-treated Olyset Plus net to be highly irritant, 88% of the mosquitoes tried to bite through the net. In addition, because most of the mosquitoes that bit through the Olyset plus net were able to take blood and further survive, these may potentially acquire one or more parasites and transmit them. In conclusion, our results suggest that the personal protection offered by ITNs is drastically reduced when the user contact the net but that the ITNs still contribute to community-wide protection by reducing short-term survival and fecundity.

Chapter 4: The resistance to deltamethrin decreases mosquito's contact irritancy but is not genetically correlated with it

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4.1 Abstract

In recent years, the efficacy of insecticide treated bed nets has been threatened by the emergence of insecticide resistance. Indeed, this latter decreases the personal protection ITNs offer by decreasing their spatial repellency and contact irritancy. Because some of the mutation responsible of resistance caused nerve insensitivity in mosquitoes, we expected these to be not only selected by killing the susceptible mosquitoes but also by the selection of the mosquitoes that are the less irritated. The aim of this study was therefore to test whether resistance was genetically correlated with contact irritancy and/or spatial repellency in mosquitoes. To do so, during 10 generations and using deltamethrin, we selected mosquitoes to be resistant, and to be strongly or weakly irritated or spatially repelled. By comparing the resistance, the irritancy and the spatial repellency of each selection-treatment, we confirm that resistance decreases the contact irritancy but did not find any genetic correlation between the resistance and the contact irritancy. Indeed, while resistant mosquitoes were less irritated, mosquitoes selected to be not irritated were as susceptible as the control ones. In conclusion, our results confirm that resistance may decrease the personal protection offered by ITNs and will help to understand the epidemiological impacts of ITNs on mosquito-borne diseases.

4.2 Introduction

Insecticide treated bed nets are one of the most important measures used for malaria control. They help to reduce malaria prevalence in several areas but particularly in the Sub-saharian part of Africa [3,7,117,118]. The insecticides used to treat the net (i.e. pyrethroids [20,138]) offer personal protection to the ITNs users by repelling mosquitoes [17,18,38], thus preventing the users from being bitten by these ones. The repellency of ITNs acts in two steps: first mosquitoes are spatially repelled by volatile molecules of the insecticides present in the surrounding of the ITNs; second, some of the mosquitoes that failed to be spatially repelled, by trying to pass or bite through the ITNs, will be irritated by the insecticide and thus these will move away before having bitten the ITNs users [71]. In addition to personal protection, the ITNs offer community protection by killing the mosquitoes that failed to be spatially repelled and/or irritated, thus reducing the number of infectious mosquitoes in the vicinity where the ITNs are used [13,19,33].

Last years, several studies reported that the efficacy of the ITNs to give valuable personal and community protection has been reduced by the emergence of pyrethroids resistance in mosquito population [33–35]. Particularly, resistance reduces the personal protection offered by ITNs by decreasing the chance that the mosquitoes are repelled. Indeed, deltamethrin-treated nets, for example, reduces the chance of mosquitoes to take blood by 72% when these are susceptible, while the reduction is "only" of 43% when mosquitoes are resistant [29]. In addition, it has been shown that a mutation in the voltage-gated sodium channel which lead to knock-down resistance (kdr) in *Anopheles gambiae* mosquitoes make them less spatially repelled and less irritated by permethrin [43,44]. In contrast, the reduction in personal protection due to resistance may be balanced by an increased community protection. Indeed, by making mosquitoes staying longer on the net, resistance to pyrethroids may increase the chance they receive a lethal dose of insecticides [29,44,45].

The evolution of resistance in malaria mosquito population is mainly attributed to the use of larvicide (for malaria control or by-product of agricultural use [36]). However, the evolution of resistance may also be triggered by the repellent action of insecticides used for IRS and ITNs [37]. Indeed, strong repellency would offer an effective personal protection but would decrease the selective pressure for resistance as mosquitoes will be less killed by the insecticide (less contact with it) [37,38,139]. Contrary, weak repellency would increase community protection and, thus, increase the selective pressure for resistance [37,39–41].

The main mechanisms by which mosquitoes resist against pyrethroids may be divided in 2 groups; metabolic or target site resistance [42]. The first one involves a higher activity or levels of one or several

enzymes enrolled in the detoxification of the pyrethroids agents. The second one regroups mutations in target sites that may cause insensitivity to insecticides. The most common one is a mutation in the voltage-gated sodium channel which causes an insensitivity to the insecticide which lead to knock-down resistance (kdr) [60].

Because the evolution of resistance seems to be linked to the insecticide's repellency [37] and because resistance reduces both spatial repellency and contact irritancy [29,43–45], we expected it to be genetically correlated to spatial repellency and/or contact irritancy. In particular, we expected the mutations in target sites that confer nerve insensitivity against insecticides to make mosquitoes less irritated [43,44] and to be selected not only by the selection of mosquitoes that survive to insecticides (resistance) but also through the selection of mosquitoes that are not irritated.

The aim of this study was therefore to evaluate if there are genetic correlations between pyrethroid resistance, contact irritancy and spatial repellency. To do so, we selected for 10 generations different lines of *Anopheles gambiae* mosquitoes to be resistant to deltamethrin, to be strongly or weakly irritated by it and to be strongly or weakly spatially repelled by it. We then compared each selective treatment for their resistance, irritancy and spatial repellency against deltamethrin and determined if resistance against insecticides provides a resistance against the 2 compounds of repellency and vice versa. We chose to work with deltamethrin because it is one of the pyrethroids that used to treat bed nets (Permanet®, Vestergaard Frandsen) and because it is known to be both spatially repel and irritate mosquitoes [71].

4.3 Methods

4.3.1 Mosquitoes

We used a Kisumu colony of *Anopheles gambiae* mosquitoes that is susceptible to deltamethrin [67]. During all the experiment, mosquitoes were maintained in an insectary at 26.5 ± 0.5 C° and 75 ± 5 % of humidity with a 12:12 hours light:dark cycle.

4.3.2 Selection

We selected *A. gambiae* mosquitoes during 10 generations to be resistant to deltamethrin, or to be strongly or weakly irritated or to be strongly or weakly spatially repelled. In addition, as control we maintained mosquitoes for 10 generations without any contact with deltamethrin. Each of the 6 selection treatments was replicated 3 times for a total of 18 lines.

We started the first generation by haphazardly selecting synchronously hatched larvae from our colony. We let them grow by 250 in 35x15x5 cm trays containing 800 mL of deionized water. Standard food levels were given to the larvae as following; 0.04ug of TetraMin Baby® fish food per larvae the day of hatching, 0.06 ug on day 1 post hatching, 0.08 ug on day 2, 0.16 ug on day 3, 0.32 on day 4 and 0.6 ug for day 5 and more. Pupae were transferred in 21x21x21 cm cages and adults were maintained with 6% sugar solution during all the procedures. Using the same larvae that we used to create our lines, during a pilot experiment, we recorded the baseline measures for the 3 following traits; resistance, contact irritancy and spatial repellency (see Measure of final traits). During the 10 generations and the measure of the final traits, all expositions or test done with deltamethrin were made using WHO standard impregnated paper with a concentration of 0.05%.

4.3.2.1 Resistance

Resistance to deltamethrin was selected following the procedure of a previous study [140]. At each generation, 4-5 day old female mosquitoes were exposed to deltamethrin-impregnated paper for a duration that may kill 50% of them 24 hours post exposure (LT50). This LT50 was determined at each generation by rearing two extra trays of 250 larvae per “resistant” line. The resulting 3-4 days old females were exposed by 20 during 5 different durations to deltamethrin, with each duration tested in duplicated for each line. This allowed us to have an estimate of the LT50 that we then used to select resistance in mosquitoes the day after. Three days after exposure to deltamethrin, the 50% of remaining females (approximatively 100 females per line) were blood fed on human arms during 5 min. Eggs were collected on day 3 post blood feeding and 1000 hatched larvae per line were used for the next generation (500 of these were used to determine the next LT50). Finally, during the selection of resistance, all expositions to deltamethrin were done using WHO-susceptibility test tubes.

4.3.2.2 Contact Irritancy

Selection for strong or weak irritancy was done using the time mosquitoes used to jump off of a deltamethrin treated paper [94]. We chose to use that measure of irritancy instead of the one proposed by Grieco and coworkers [141]. Indeed, with their system, it is not possible to completely exclude that some of the mosquitoes were only spatially repelled.

For each line, 6-7 days old female mosquitoes were blood fed on human arms for 5 minutes and the day after, 150 of them were transferred in individual 120 ml plastic cups. Two days post blood feeding, we add fresh deionised water in the cups in order to allow females to lay eggs. During days 3 and 4 post blood meal, females that laid eggs were tested individually for their irritancy against deltamethrin.

To do so, mosquitoes were transferred in the resting part of a WHO-susceptibility test tube. After 2 minutes of acclimatization, we gently blew them in the tube that contained the deltamethrin-impregnated paper and the time until their first jump was recorded; i.e. the time from landing on the paper up to flying in an attempt to escape from the insecticide paper [94]. The eggs of the 50% of mosquitoes that jumped off of the paper the faster (approximatively 70 females per line) were selected for the lines that represented strongly irritated mosquitoes ("irritated" lines). Conversely, the eggs of the 50% that stay the longest time on the deltamethrin were selected for the lines that represented the weakly irritated mosquitoes ("not irritated" lines). After hatching, a total of 500 larvae were selected equally from each selected female and used for the next generation. We chose to blood feed the females before the test for irritancy in order to avoid any maternal effects due to the insecticide.

4.3.2.3 Spatial repellency

Spatial repellency was measured as the preference of mosquitoes to blood feed in the presence of deltamethrin or not, using a modified high-throughput screening system (HISS) [141,142]. We build our two-way choice apparatus using WHO-susceptibility test tubes and it consisted in an assembly of 3 test tubes (Fig. 1). Two external tubes, which will be called the test tubes, were connected with a central one. Inside each test tube, a WHO paper (impregnated with deltamethrin or untreated) was placed between the walls of the test tube and an extra tube (previously perforated and covered with a piece of non-treated net) which was inserted directly in the test tube (Fig.1). By doing so, we created a passage for the mosquitoes in which they could not contact the insecticide but where there were insecticide's volatiles. This allowed us to measure the spatial repellency of deltamethrin and to completely exclude the contact irritancy as mosquitoes were not able to contact the treated paper. During the measure of spatial repellency, mosquitoes were placed by 20 in the central tube and we let them acclimate for 2 min. After acclimatization, the plastic doors that connected the central tube to the test tubes were opened and mosquitoes were allowed to choose between the treated tube or the control tube. To make mosquitoes choosing one side, we attracted them by putting an arm at the end of each test tube without contacting it. Indeed, we wanted to avoid that mosquitoes take blood. In addition, to avoid any side preference, the side of the tube containing the deltamethrin was alternate between each test. After the 6 minutes of test, the plastic doors were closed and the number of mosquitoes in each tube were counted. Mosquitoes that were found in the tube with the deltamethrin were used to make the "not spatially repelled" lines whereas the one found in the tube with the control paper were used to make the "spatially repelled" lines. The selected mosquitoes were placed in their respective cages and blood fed on human arms 24 hours after the tests. The resulting larvae were used for the next generation.

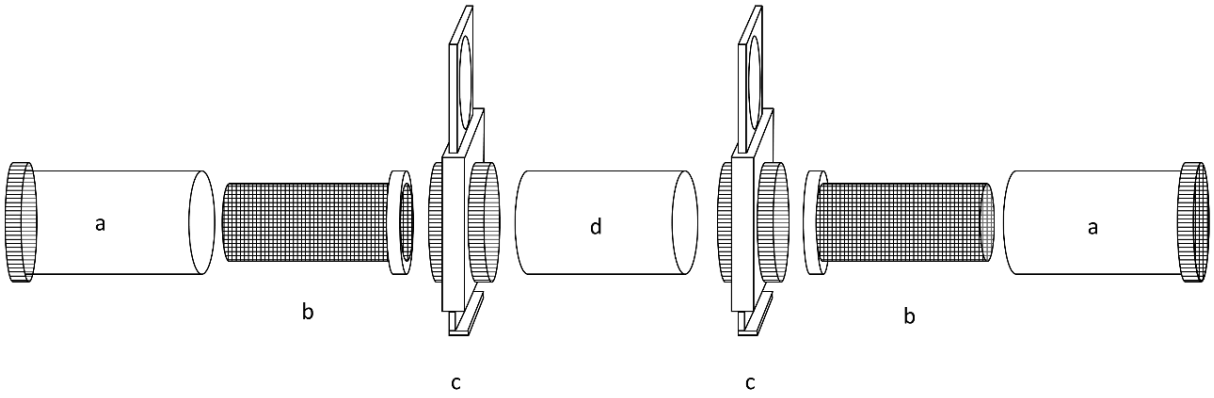

Figure 1: Schematic drawing of the two-way choice apparatus used to test spatial repellency. a Test tubes; b inserted perforated tubes covered with untreated net; c plastic doors; d central tube. Deltamethrin-treated paper or untreated paper were inserted between the walls of the test tubes and the net of the inserted perforated tube.

4.3.2.4 Control

Three control lines that were never exposed to deltamethrin were also maintained for 10 generations. To avoid any effect of the WHO-susceptibility test tubes during the selection procedure, 3-4 days old females from each control line were exposed by 20 to untreated paper for the same duration than the correspondent LT50 used to selected resistance. Three days post exposure, females were blood fed on human arms and the resulting larvae were used for the next generation.

4.3.3 Measure of final traits

No selection was made on the mosquitoes of the 10th generation in order to relax the selective pressure and decrease maternal effects. The measures of insecticide resistance, contact irritancy and spatial repellency of each line were made in two blocks on the females from the 11th generation. To do so, for the first block, females were blood fed on day 3 post emergence and for the second, females were blood on day 20 post emergence. Except for the measure of LT50, mosquito larvae were reared individually in 12-well plates with a standard fish food level as described above. For the measure of LT50, due to the high number of mosquitoes needed, larvae were reared by 250 in trays with the same standard level of food. The same procedure was followed for the pilot tests. Overall, for the final tests, for each block, 6480 larvae were reared in 12-well plates (360 per line) while for the LT50, 9000 larvae (500 per line) were reared in trays for the first block and only 4500 were available for the second block (250 line).

4.3.3.1 Resistance

At the beginning (pilot experiment) and at the end of the selection, resistance of each line was measured using two traits: the LT50 and the resistance after 10 min of exposition to deltamethrin.

For the measure of LT50, female mosquitoes of each line were exposed in WHO-susceptibility test tubes to 0.05% of deltamethrin for 5 different durations; 0.5, 1, 2, 4 and 8 minutes. For the pilot experiment, 3 replicates of 15 mosquitoes for each duration were exposed to deltamethrin and used to build the time-response curve. At the end of the selection, during the first block, two replicates of 15 mosquitoes per duration were used for each line while we were able to have only one replicate per duration and line for the second block. The mortality of mosquitoes 24 hours post exposure was recorded and the time-response curve and the LT50 of each line were analysed. We thus built the time-response curve for each line using about 45 mosquitoes per durations and lines, for a total of about 4050 mosquitoes.

For the second measure of resistance, mosquitoes of each line were exposed by approximatively 10 during 10 minutes to 0.05% of deltamethrin using WHO-susceptibility test tubes. For the pilot experiment, 8 replicates of mosquitoes were exposed to deltamethrin while 5 were exposed to untreated paper as control. At the end of the selection, during the first block, 4 replicates per line were exposed to deltamethrin, while 2 replicates per line were exposed to untreated paper. During the second block, due to high mortality during larval development, only 3 replicates per line were exposed to deltamethrin and 2 replicates per line to control paper. We therefore tested the resistance of about 110 mosquitoes per line for a total of about 1980 females. Resistance to the 10 min of exposure to deltamethrin was assessed by evaluating the mortality in each replicates 24 hours post exposure.

4.3.3.2 Contact Irritancy

For both pilot and final tests, the tests for irritancy were done following the same procedure than for the selection for strong or weak irritancy (see above). However, this time, we not only recorded the time until their first jump but also the number of times they jumped within one minute. For the pilot tests, 30 five days old female mosquitoes were tested on untreated paper while 42 were tested on deltamethrin-impregnated paper. For the final measurements, during the first block, 5 days post emergence, for each line and during 3 following days, 24 mosquitoes were tested individually on deltamethrin-impregnated paper and as control, 15 were tested on untreated paper (8 mosquitoes on deltamethrin and 5 on untreated paper per day and per line) for a total of 702 tested mosquitoes. For

the second block, also 5 days post emergence, during 3 followings days and for each line, this time 21 mosquitoes were tested on deltamethrin-impregnated paper whereas 15 ones were tested on untreated paper, for a total of 648 tested mosquitoes.

4.3.3.3 Spatial repellency

For pilot and final tests, spatial repellency was measured by following the same procedure used to select the mosquitoes for or against repellency (see above). During the pilot experiment, 5 replicates of approximately 10 mosquitoes were tested for their choice between 2 untreated paper while 9 replicates had to choose between deltamethrin-impregnated paper or untreated paper. For the final measurements, during the first block, we tested mosquitoes by approximately 10 in 4 replicates during which mosquitoes had to choose between untreated or deltamethrin-impregnated paper. As control, we also tested 2 replicates per line that had to choose between two untreated paper. For the second block, we were able to test 2 replicates of approximatively 10 mosquitoes per line that had to choose between deltamethrin or untreated paper and 2 replicates per lines that had to choose between two untreated paper. For each test, the number of mosquitoes found in each tube was recorded and the resulting proportion of repelled mosquitoes (the ones that chose the tube containing the control paper) was used to evaluate the spatial repellency of each line.

4.3.4 Statistical analysis

For all analysis, the best model was found by model comparisons and non-significant interactions were removed. The significance of the effects was tested using type 2 anova when no significant interactions were in the model and using type 3 anova when at least one interactions in the model was significant (both type of anova coming from the *car* library in R [97]). All analysis and graphs were performed using the software R (version 3.5.0) [98] and its Rstudio interface (version 1.1.453) [99].

4.3.4.1 Resistance

Resistance of the mosquitoes was assessed by comparing the LT50 values and the proportion of mosquitoes that died 24 hours after 10 min exposure to deltamethrin between the selection-treatments.

To compare the LT50 values between the selection-treatments we first built time-response model using the *drm* function (*drc* library in R [127]). We used a 2 parameters log-logistic function with higher and lower limits for time-mortality curves set to 1 and 0 respectively. The statistical comparisons of the LT50 values between each selection-treatment was done using the *EDcomp* function of the *drc*

package. Because in that analysis we were not able to control for the effect of the blocks and the mosquito's lines (replicates) of each treatments, we run a second analysis using a general linear mixed model (GLMM) with a binomial distribution from the *lme4* library [100]. In this analysis, the proportion of dead mosquitoes was set as the response variable with the time of exposure as covariate, the selection-treatments and the blocks as fixed factors and the mosquito's lines and the replicates of exposure (test identities) as random factors. Comparisons between the 6 different treatments (control, resistant, not irritated, strongly irritated, not spatially repelled, strongly spatially repelled) were done using a post-hoc Tukey test that adjusted the p-values for multiple comparisons.

We analysed the proportion of mosquitoes that died after 10 min exposure on deltamethrin-impregnated paper or untreated paper using a GLMM. We first compared the mosquito's mortality of the deltamethrin-impregnated paper and the untreated nets separately. In both analysis the selection-treatments and the block identity were set as fixed factors while the mosquito's lines and the replicates of exposure were set as random factors. Comparisons of survival between the 6 different treatments were done using a post-hoc Tukey test. Finally, a full analysis that groups the data of each paper was run using a GLMM with a binomial distribution. The type of paper, the selection-treatments and the block identity were set as fixed factors while, as for the two first analysis, the mosquito's lines and the replicates of exposure were set as random factors.

4.3.4.2 Contact irritancy

The contact irritancy of each paper, deltamethrin-impregnated or untreated, was evaluated by analysing the time mosquitoes took until their first jump and the jump frequencies of the mosquitoes that jumped. For both measures, we first analysed the effect of each paper separately and second run a full analysis that regroups the data from each paper. In addition, comparisons of irritancy of each paper between the 6 different treatments were done using a post-hoc Tukey test.

The time mosquitoes took until their first jump was analysed using a Cox proportional hazards regression mixed model (*coxme* library in R [95]) with the selection-treatments and the blocks as fixed factors, the day of test as covariate and the mosquito's lines as random factors. For the full analysis that combined the data from each paper, in addition to these explanatory variables, the type of paper was added as fixed factor.

The jump frequency of mosquitoes was analysed using a linear mixed effect model (LMM) (*lme4* library) with the same parameters than for the analysis of the time mosquitoes took until their first jump. Again, in the full analysis pooling the data of each paper, we add the type of paper as fixed factor. For all analysis of jump frequencies, normality of the residuals was visually assessed.

4.3.4.3 Spatial repellency

To first analyse if mosquitoes had a preference for one of the sides of our high-throughput screening system, we used a repeated G-test of goodness-of-fit on the pooled data of each selection-treatment that compare the proportion of mosquitoes that chose the right side against a random choice (p=0.5). To remember, during that tests, mosquitoes had the choice between two untreated papers. A second analysis using a GLMM with binomial distribution was run to evaluate whether the preference for one side was affect by the selection-treatments and if it was different between the blocks. In that analysis, the proportion of mosquitoes choosing the right side was set as the response variable with the selection-treatments and the block identities as fixed factors and the mosquito's line and the identities of tests (replicates) as random factors.

The analysis of the deltamethrin repellency was similar to the analysis for a side preference. Indeed, using a G-test of goodness-of-fit, we first assessed whether deltamethrin was spatially repellent by comparing the proportion of mosquitoes choosing the tube with the untreated paper instead of the deltamethrin-impregnated paper against a random choice (p=0.5). Second, a GLMM, with the same parameter than for the analysis of side preference, was used to compare the spatial repellency between the selection-treatments and blocks.

4.3.4.4 Genetic correlations

As we were not able to select strongly or weakly spatially repelled mosquitoes, we were only able to test if there was a genetic correlation between resistance and irritancy. We tested whether the two traits were genetically correlated using a Pearson correlation tests. It tested whether mean value of resistance and contact irritancy of each line were significantly correlated. As a measure of resistance, we used the LT50 values of each line, while we used both time until first jump and the jump frequencies as measure of irritancy. The analysis was done in two steps; first, we tested if resistance and irritancy were correlated using the mean values of the resistant and control lines and second, the same tests were done using this time the values of the lines selected to be strongly or not irritated.

4.4 Results

A summary of the mean value of each selection-treatment for the measures of resistance, contact irritancy and spatial repellency are presented in Table 1. A total of 8686 female mosquitoes were used during the two blocks, 3487 for the measure of LT50, 2049 for the measure of resistance after 10 min

of deltamethrin exposure, 1350 for the measure of contact irritancy and 1801 for the measure of spatial repellency.

Table 1: Summary of the mean value of resistance, contact irritancy and spatial repellency for each selection-treatment.

Selection-treatments	Resistance				Contact irritancy				Spatial repellency	
	LT50 [min]		10min exposure [%]		Time to 1st jump [s]		Jump frequency [j/s]		Proportion repelled [%]	
	Mean	*SE*	*Mean*	*95% i.c.*	*Mean*	*SE*	*Mean*	*SE*	*Mean*	*95% i.c.*
Control	0.95	0.07	97.7	80.8-99.8	20.29	1.8	0.16	0.006	42.2	22.8-64.3
Resistant	4.6	0.31	77.1	55.9-90	36.07	1.99	0.1	0.005	52.7	31.3-73.2
Irritated	1.3	0.11	90.7	71.3-97.4	14.99	1.63	0.22	0.009	45.4	25.3-67.1
Not irritated	1.01	0.08	96.3	78.9-99.5	31.74	2.02	0.12	0.006	43.5	23.9-64.6
Sp. repelled	0.99	0.07	98.3	81.8-99.9	20.28	1.71	0.16	0.006	42.5	23-64.6
Not Sp. repelled	0.87	0.08	98.0	81.4-99.8	20.43	1.75	0.16	0.005	47.6	27-68.9

Because we found no difference in resistance [LT50 (χ^2=1.14, df=1, p=0.29) and resistance after 10 min exposure to deltamethrin (χ^2=0.01, df=1, p=0.93)], contact irritancy [time to first jump (χ^2=0.19, df=1, p=0.73) and jump frequency (F=0.01, df=1, p=0.93)] and spatial repellency [choice for control paper (χ^2=0.0003, df=1, p=0.98)] between the pilot experiment and the lines maintained during 10 generation as control, we chose to use the latter ones as control in the further analysis. Thus, any difference between the control lines and the others would mean some traits were selected.

4.4.1 Resistance

4.4.1.1 LT50

The LT50 of our selection-treatments ranged between 0.87 to 4.6 minutes (Table 1). Summary of the multiple comparisons of the LT50 values of each selection-treatment can be found in Table 2 (post-hoc Tukey test). Briefly, the LT50 of the mosquitoes selected to be resistant was significantly higher than all the other selection-treatment (Fig.2A). Mosquitoes selected to be strongly irritated had also a significantly higher LT50 compared to the control, spatially repelled, not spatially repelled and the not irritated lines. These later selection-treatments were found to not statistically diverge for their LT50.

Table 2: Results of multiple comparisons of LT50 values and mosquito's mortality 24 hours after 10 min exposure to deltamethrin (post-hoc).

Comparisons	LT50		10 min exposure	
	t-value	*p-value*	*z-value*	*p-value*
Control-Resistant	38.4	<0.001***	5.2	<0.001***
Control-Irritated	3.4	<0.001***	2.8	0.048*
Control-Not irritated	58.9	0.56	0.8	0.97
Control-Sp. repelled	38.8	0.7	0.4	0.99
Control-Not sp. repelled	74.4	0.46	0.4	0.99
Resistant-Irritated	23.4	<0.001***	3.8	0.002**
Resistant-Not irritated	34	<0.001***	5.2	<0.001***
Resistant-Sp. repelled	8	<0.001***	5.3	<0.001***
Resistant-Not sp. replled	38.4	<0.001***	5.2	<0.001***
Irritated-Not irritated	2	0.047*	2.3	0.18
Irritated-Sp. repelled	2.2	0.02*	3.1	0.02*
Irritated-Not Sp. repelled	2.8	0.006**	3.1	0.02*
Not irritated-Sp. repelled	21.4	0.83	1.2	0.82
Not irritated-Not sp. repelled	1.18	0.24	1.1	0.85
Sp. repelled-Not sp. repelled	1.2	0.22	0.05	1

A second analysis of the dose-response curves gave similar results. The proportion of dead mosquitoes logically depended on the duration of exposure to deltamethrin (χ^2=76.23, df=1, p<0.001). The selection-treatment from which mosquitoes originated significantly determined the mosquito's probability of dying (χ^2=59.35, df=5, p<0.001) (Fig. 2A). The mosquitoes selected to be resistant had a smaller chance of dying compared to the other selection-treatment, the mosquitoes selected to be strongly irritated had an intermediate chance of dying, and the remaining selection-treatments had the same chance of dying (see LT50 values in Table 1). In addition, the proportion of dead mosquitoes was significantly influence by the interaction between the duration of exposure (dose) and the selection-treatment (χ^2=34.88, df=5, p<0.001). Finally, although the proportion of dead mosquitoes was significantly different between the two blocks (χ^2=4.51, df=1, p=0.03), the effect of the treatment remained the same within the blocks (χ^2=5.11, df=5, p=0.4).

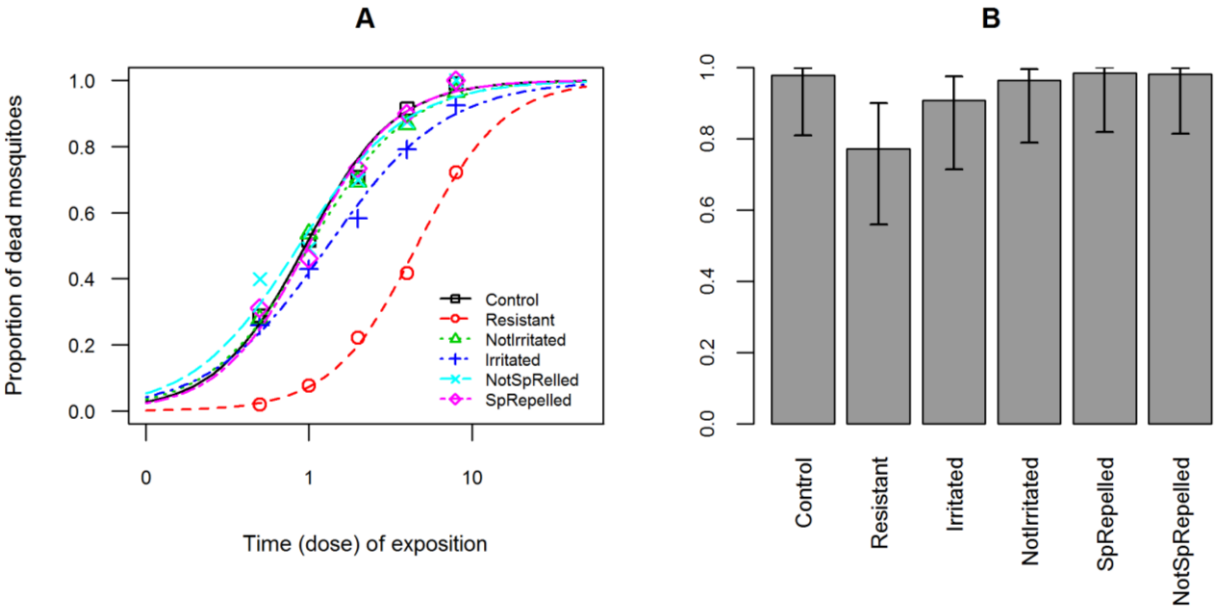

Figure 2: Results of the tests for resistance. ***A****: Logistic regression of the proportion of dead mosquitoes in function of the duration of exposure to deltamethrin. The different selection-treatment are highlighted by the colors.* ***B:*** *Proportion of dead mosquitoes 24 hours after a 10 min exposure to deltamethrin in function of the selection-treatments. Bars represent the 95% interval of confidence.*

4.4.1.2 Resistance to 10 min of deltamethrin exposure

The proportion of dead mosquitoes 24 hours after 10 min exposure on the untreated paper range between 0 to 2.8% and was not statistically different between the selection-treatments (χ^2=0.79, df=5, p=0.98) and between the two blocks (χ^2=1.94, df=1, p=0.16).

Mosquito's mortality 24 hours post 10 min exposure to 0.05% of deltamethrin was between 77.1 % and 98.3 % (Table 1). The selection treatment significantly influenced the proportion of dead mosquitoes (χ^2=82.2, df=5, p<0.001). In particular, the mosquitoes selected to be resistant had a better survival compared to all 5 other selection-treatments (Fig. 2B). The survival of the mosquitoes selected to be irritated was significantly higher than the one of the control, the spatially repelled or not spatially repelled lines but not different from the not irritated lines. The survival did not differ between the mosquitoes selected to be or not spatially repelled, the one selected to be not irritated and the control ones (see Table 2 for multiple comparisons). Finally, the mosquito's mortality after 10 min exposure to deltamethrin was not different between the two blocks (χ^2=1.76, df=1, p=0.18).

In the full analysis that pooled the data of the two type of paper (untreated and deltamethrin), we found the deltamethrin increasing the risk of dying after 10 min exposure by a factor of 62.4 (χ^2=400.86, df=1, p<0.001). The proportion of dead mosquitoes was significantly different between the

selection-treatments (χ^2=75.99, df=5, p<0.001) but with no effect of the interaction between the type of paper and the selection-treatments (χ^2=7.17, df=5, p=0.21). Thus, it suggests that the mosquito's probability of dying is increased by deltamethrin independently of the selection-treatment from which mosquitoes originated. Finally, the proportion of dead mosquitoes did not differ between the two blocks (χ^2=0.57, df=1, p=0.45).

4.4.2 Contact Irritancy

We used two ways to measure contact irritancy of mosquitoes to deltamethrin; the time until their first jump and the jump frequencies of the mosquitoes that jumped. Both measures were recorded on untreated paper or deltamethrin-impregnated paper. Summary of the post-hoc comparisons of the time to first jump and the jump frequency for each type of paper (untreated or deltamthrin-impregnated) can be found in Table 3.

Table 3: Results of the post-hoc tests (Tukey) that compare the time to mosquito's first jump and the mosquito's jump frequency between the selection-treatment when mosquitoes were exposed to untreated paper.

Comparisons	Untreated paper				Deltamethrin-impregnated paper			
	Time to 1st jump		Jump frequency		Time to 1st jump		Jump frequency	
	z-value	*p-value*	*z-value*	*p-value*	*z-value*	*p-value*	*z-value*	*p-value*
Control-Resistant	1	0.92	0.4	0.99	5.7	<0.001***	5.7	<0.001***
Control-Irritated	3.4	0.01*	5.9	<0.001***	3	0.04*	5.6	<0.001***
Control-Not irritated	1.5	0.7	1.1	0.86	4.3	<0.001***	4	<0.001***
Control-Sp. repelled	0.2	0.99	1.5	0.63	0.2	1	0.3	0.99
Control-Not sp. repelled	0.11	1	1	0.93	0.4	0.99	0.2	0.99
Resistant-Irritated	4.3	<0.001***	6	<0.001***	8.4	<0.001***	10.8	<0.001***
Resistant-Not irritated	0.5	0.99	1.5	0.68	1.5	0.68	1.7	0.52
Resistant-Sp. repelled	1.2	0.82	1.9	0.4	5.5	<0.001***	5.4	<0.001***
Resistant-Not sp. replled	0.9	0.95	1.3	0.77	5.3	<0.001***	5.4	<0.001***
Irritated-Not irritated	4.7	<0.001***	4.2	<0.001***	7.11	<0.001***	9.3	<0.001***
Irritated-Sp. repelled	3.1	0.02*	4.3	<0.001***	3.2	0.02*	6	<0.001***
Irritated-Not Sp. repelled	3.5	0.007**	4.8	<0.001***	3.4	0.01*	5.8	<0.001***
Not irritated-Sp. repelled	1.7	0.54	0.3	1	4.1	<0.001***	3.7	0.003**
Not irritated-Not sp. repelled	1.3	0.76	0.2	1	3.9	0.001***	3.8	0.002**
Sp. repelled-Not sp. repelled	0.4	0.99	0.6	0.99	0.2	0.99	0.1	0.99

4.4.2.1 Irritancy of untreated paper

When tested on the untreated paper, mosquitoes took in average 37.5 seconds to jump and they jump about 5.64 times per minute. The time until first jump was significantly influence by the selection treatment (χ^2=30.5, df=5, p<0.001) with, in particular, the mosquitoes selected to be irritated that were taking significantly less time to jump than the 5 other lines which did not statistically differ from each other (Table 3) (Fig. 3A). The time to first jump on untreated paper was not influence by the block (χ^2=0.6, df=1, p=0.44) or the day of test (χ^2=1.74, df=1, p=0.19).

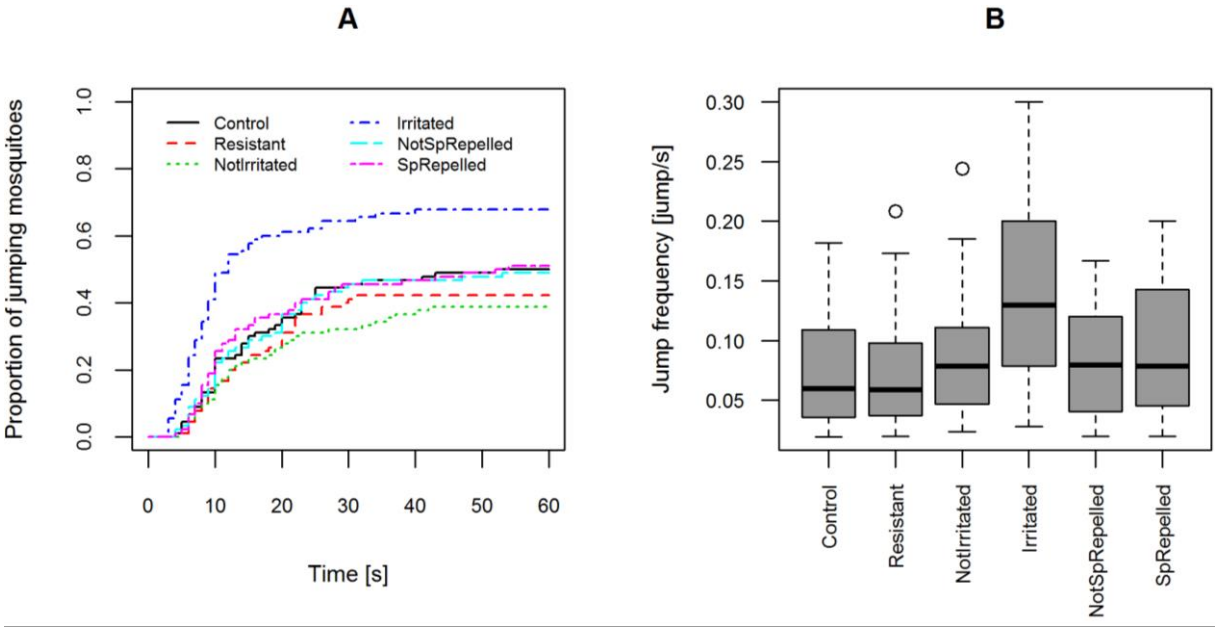

Figure 3: Contact irritancy of the untreated paper. ***A:*** *Cumulative proportion of jumping mosquitoes in function time. Colors and type of lines represent the different selection-treatments.* ***B:*** *Jump frequency in function of the selection-treatments. For each treatment, black lines represented the median jump frequency.*

Similarly, the jump frequencies of mosquitoes tested on untreated paper was significantly influence by the treatment (χ^2=53.34, df=5, p<0.001) with the irritated lines having a significantly higher jump frequencies than the 5 other lines, for which the jump frequency was not different (Table 3) (Fig. 3B). Even if the mean jump frequency of mosquitoes was globally higher during the second block (χ^2=8.39, df=1, p=0.004), the effect of the selection treatment was the same within the two blocks (i.e. no effect of the interaction block:selection-treatment, χ^2=3.41, df=5, p=0.64). Finally, the mosquito's jump frequencies were not statistically different within the 3 days of test (χ^2=3.67, df=1, p=0.06).

4.4.2.2 Irritancy of deltamethrin-impregnated paper

In average, mosquitoes took 24 seconds to jump off of the 0.05% deltamethrin paper and jumped 9.4 time per minute. The time until first jump was significantly affected by the selection treatment (χ^2=94.95, df=5, p<0.001) (Fig. 4A). In particular, mosquitoes selected to be resistant and the ones selected to be not irritated (which were not statistically different from each other) significantly took more time to jump off of the deltamethrin compared to the other selection-treatments. In addition, the mosquitoes selected to be strongly irritated took significantly less time compared to the control, the spatially repelled and the not spatially repelled lines. The 3 latter selection-treatment did not differ in the time they took to jump off of the deltamethrin (see summary of comparisons in Table 3). Although the time mosquitoes took to jump off of the deltamethrin was higher in the first blocks (χ^2=8.09, df=1, p=0.004), the effects of the selection-treatments were the same within the two blocks

(i.e. no effect of the interaction block:selection-treatment, χ^2=2.45, df=5, p=0.78). The time mosquitoes took to jump did not differ between the 3 days of test (χ^2=0.05, df=1, p=0.82).

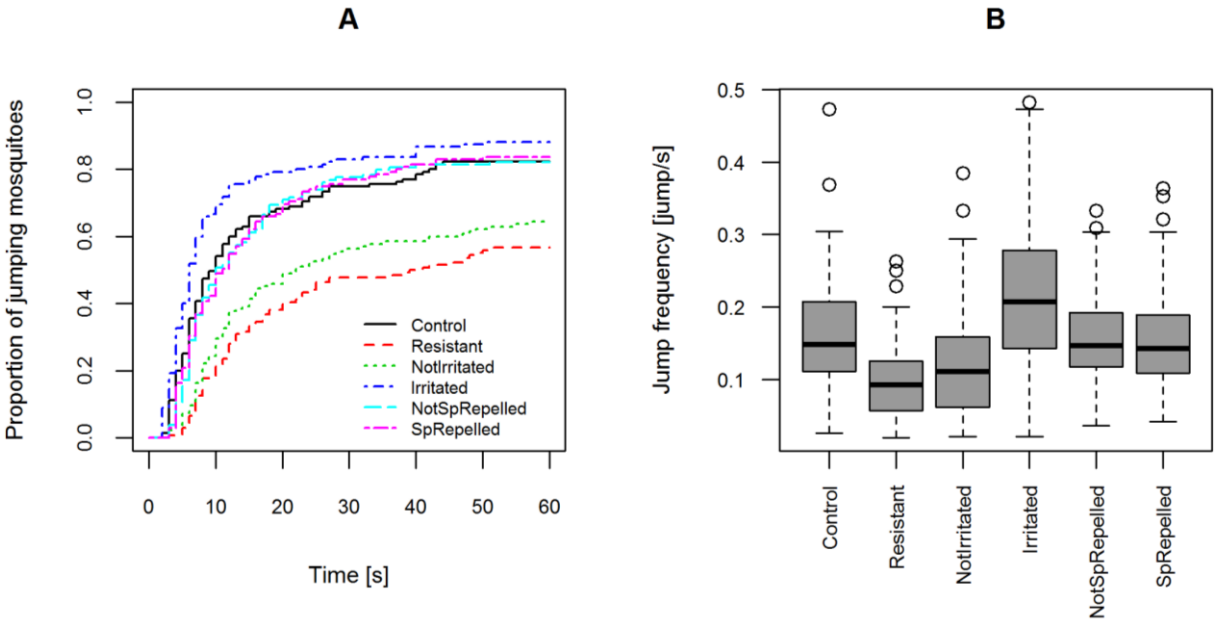

Figure 4: Contact irritancy of the deltamethrin-impregnated paper. ***A:*** *Cumulative proportion of jumping mosquitoes in function time. Colors and type of lines represent the different selection-treatments.* ***B:*** *Jump frequency in function of the selection-treatments. For each treatment, black lines represented the median jump frequency.*

The jump frequencies of mosquitoes exposed to deltamethrin were significantly influenced by the selection-treatment (χ^2=145.72, df=5, p<0.001) (Fig. 4B). Similarly to the analysis of the time mosquitoes took to jump, the jump frequencies were statistically lower for the resistant and not irritated lines compared to the other treatments (with no difference between these first two treatments). In addition, the mean jump frequency of the mosquitoes selected to be irritated was significantly higher compared to the control, the spatially repelled and the not spatially repelled lines. The 3 latter selection-treatment did not differ in their jump frequencies when exposed to deltamethrin (see summary of comparisons in Table 3). The mosquito's jump frequencies did not differ between the two blocks (χ^2=0.68, df=1, p=0.41) but was influence by the day of test (χ^2=15.75, df=1, p<0.001), with the jump frequency decreasing with mosquito's age.

4.4.2.3 Full analysis of contact irritancy

In the analysis that pooled the data from the two type of paper (untreated or deltamethrin), we found the deltamethrin to decrease the time to first mosquito jump with a factor of 1.6 (χ^2=121.78, df=1, p<0.001) while it increased the jump frequencies with a factor of 1.7 (χ^2=48.95, df=1, p<0.001).

We found a general effect of the selection-treatment on the time mosquitoes took to jump off of the paper (χ^2=117.08, df=5, p<0.001) but, however, did not find any effect of the interaction between the type of paper and the selection-treatment (χ^2=7.61, df=5, p=0.18). Thus, we found the deltamethrin to decrease the time mosquitoes took to jump independently of their selection-treatments. The time to first jump was slightly different within the 2 blocks (χ^2=3.93, df=1, p=0.047) but there was no effect of the day of test (χ^2=0.37, df=1, p=0.54).

The analysis of the jump frequencies gave similar results. Indeed, we found a general effect of the selection-treatment (χ^2=30.99, df=5, p<0.001), but this time we also found a significant effect of the interaction between the selection-treatment and the type of paper (χ^2=18.45, df=5, p=0.002), confirming the results found with the analysis of each paper done separately (Fig. 3B and Fig. 4B). In this full analysis of jump frequency, we found no effect of the block (χ^2=3.58, df=1, p=0.06) but a decrease in jump frequency was found within the 3 days of test (χ^2=7.89, df=1, p=0.005).

4.4.4 Spatial repellency

When giving the choice between two untreated paper, the preference of mosquitoes for one side of the two-way apparatus did not differ between the selection-treatments (χ^2=0.76, df=5, p=0.98). There was also no difference in that preference between the two blocks (χ^2=0.07, df=1, p=0.79). In addition, a repeated G-test of goodness-of-fit pooling the data of all selection-treatment showed strong support for no side preference [total-G=16.91, df=74, p=1, pooled-G=0.2, df=1, p=0.66, with no heterogeneity among the replicates (G = 16.71, df = 71, p=1)].

The repellency of deltamethrin, represented by the proportion of mosquitoes choosing to blood feed in the presence of an untreated paper instead of deltamethrin-impregnated paper, did not differ between the selection-treatment (χ^2=5.33, df=5, p=0.38) and the two blocks (χ^2=0.002, df=1, p=0.96). However, a repeated G-test of goodness-of-fit showed that deltamethrin spatially repelled mosquitoes during the tests, with 45.65 % (95% c.i., 42.15 to 48.67%) of mosquitoes choosing the tube with the untreated paper [total-G=65.97, df=108, p<0.001, pooled-G=7.86, df=1, p=0.005, with no heterogeneity among the replicates (G=58.11, df=107, p=1)] (Fig. 5).

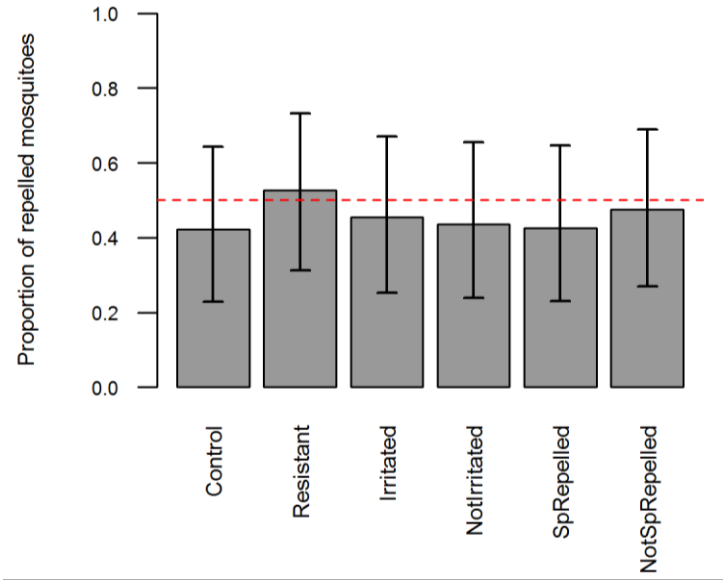

Figure 5: Proportion repelled mosquitoes in function of their selection-treatment when facing a deltamethrin-impregnated paper and an untreated paper. Bars represent the 95% interval of confidence. The red dashed line represents mosquitoes that would have no preference (p=0.5).

4.4.5 Genetic correlations

As we were no table to select mosquitoes to be strongly or weakly spatially repelled, we only tested whether resistance and contact irritancy were genetically correlated.

Using the control lines and the lines selected to be resistant, we found the measures of LT50 to be positively correlated with the time mosquitoes took to jump off the deltamethrin paper ($t=23.27$, $df=4$, $p<0.001$, $R^2=0.99$) (Fig. 6A) and to be negatively correlated with the jump frequency ($t=-4.89$, $df=4$, $p=0.008$, $R^2=0.86$) (Fig. 6B). This indicated that the more resistant the mosquitoes were, the less they were irritated.

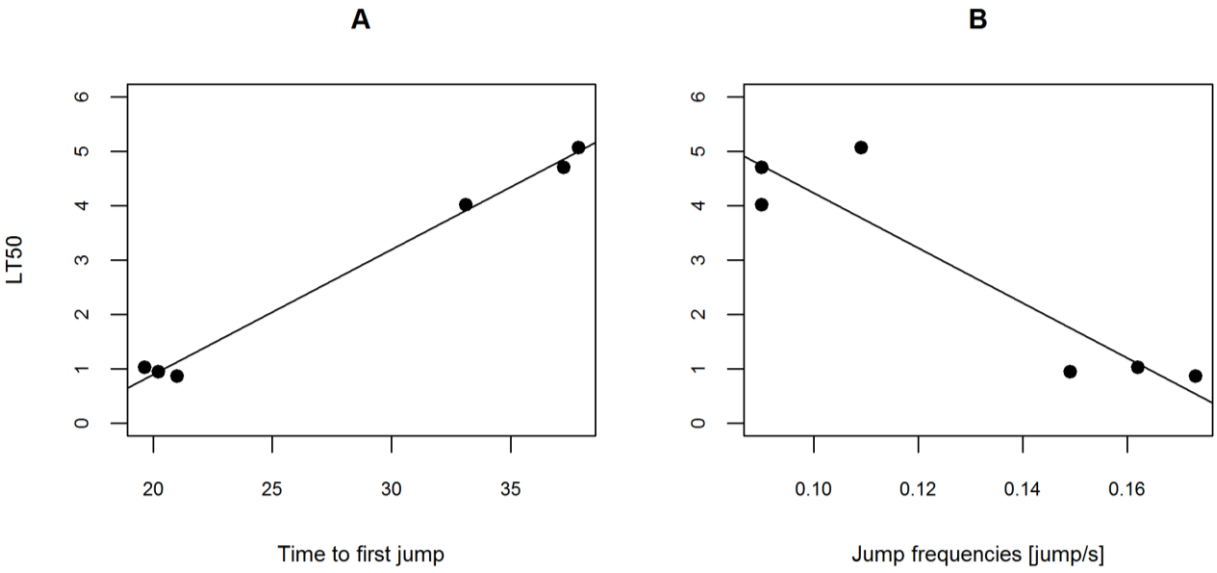

Figure 6: Correlation between the degree of resistance (represented by the LT50) and the degree of irritancy built with the measures of the resistant and control lines. ***A:*** *Correlation between LT50 and the time mosquitoes took to jump.* ***B:*** *Correlation between the LT50 and the mosquito's jump frequencies. For both graphs, the lines represent the results of a linear regression.*

In the other way, by using the line selected to be strongly irritated and the ones selected to be not irritated, we found the LT50 values to be marginally negatively correlated with the time mosquitoes took to jump off the deltamethrin (t=-2.62, df=4, p=0.06, R^2=0.63) (Fig. 7A) and to be marginally positively correlated with their jump frequencies (t=2.86, df=4, p=0.046, R^2=0.67) (Fig. 7B). These results indicate that the most irritated the mosquitoes were, the most they were resistant.

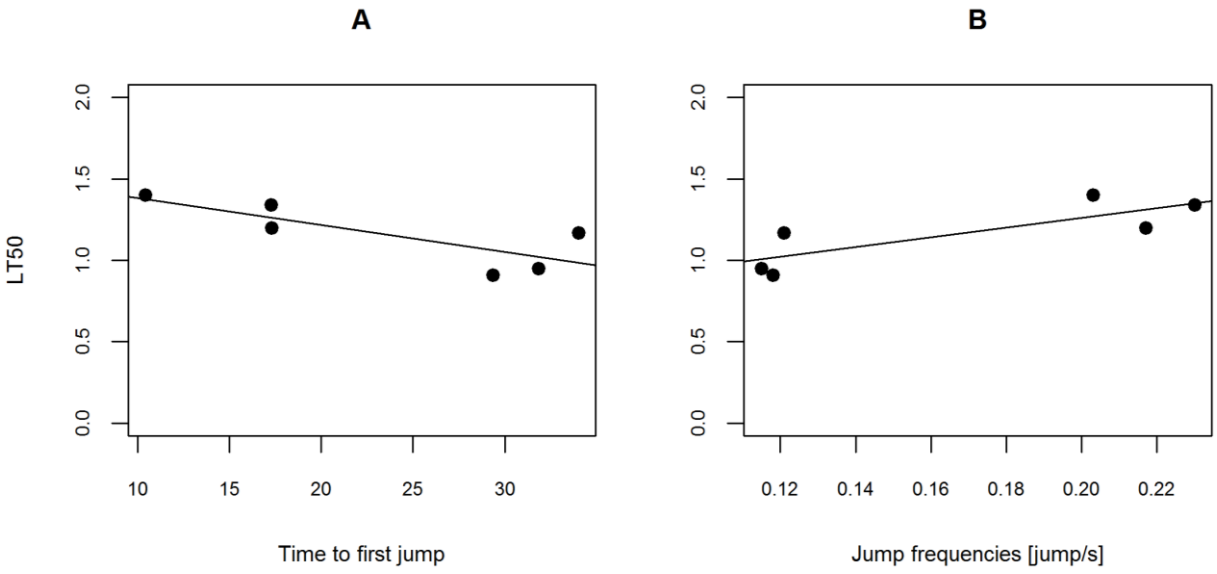

Figure 7: Correlation between the degree of resistance and the degree of irritancy built with the measures of the "irritated" and "not irritated" lines. ***A:*** *Correlation between LT50 and the time mosquitoes took to jump.* ***B:*** *Correlation between the LT50 and the mosquito's jump frequencies. For both graphs, the lines represent the results of a linear regression.*

4.5 Discussion

During this evolutionary experiment, we were able to select mosquitoes to be: 1) partially resistant to deltamethrin, 2) strongly irritated and 3) weakly irritated by the deltamethrin. We were however not able to select them to be strongly or weakly spatially repelled. Despite so, we confirm that the resistance decreases the contact irritancy of mosquitoes [43,44]. However, although resistance decreases mosquito's irritancy, the mosquitoes selected to be not irritated were as susceptible as the control lines, suggesting there was no genetic correlation between these two measures.

Even if almost 5 times higher than at the beginning, the resistance of our mosquitoes to deltamethrin after 10 generation of selection was not as high as Chareonviriyaphap and coworkers found after the same number of generations in *A. minimus* [140]. Despite so, we were able to confirm the results of previous studies showing that resistance decreased the contact irritancy of the mosquitoes [43,44]. Indeed, we confirm the resistance to decrease the number of jump mosquitoes are doing when exposed to the insecticides but in addition, we also found that it prolonged the time they used to jump off of the deltamethrin paper. Because of this reduced irritancy, we expected that we selected a target site resistance rather than a metabolic one. Indeed, because the latter one seems to be time-lagged as it mostly helps mosquitoes to detoxify the insecticides [42], the target site resistance that confer nerve insensitivity seems to be more appropriate to reduce mosquito's irritancy. This hypothesis is also supported by the results of a first study that found that kdr resistant *A. gambiae* were not irritated by permethrin and deltamethrin while other resistant *A. arabiensis* and *A. funestus* mosquitoes that presents a metabolic resistance were equally irritated than susceptible mosquitoes [43].

The mosquitoes selected to be not irritated (i.e. jumping the slower from the paper and having the smaller jump frequencies) were as susceptible to deltamethrin as the control mosquitoes. More surprisingly the mosquitoes selected to be strongly irritated had a higher resistance than the not irritated and the control mosquitoes. We explained this latter result by the fact they may have passed most of the time flying instead of contacting the deltamethrin. On the one hand, it suggests that the genes responsible for strong irritancy may offer a behavioural resistance to insecticides, on the other, because strongly irritated mosquitoes were jumping faster and more frequently also on the untreated paper, it suggests we selected the mosquitoes that were the most *excited*. In particular, we expected to have selected the mosquitoes that were the most sensible to human odour rather than the most irritated. Indeed, because before being exposed to deltamethrin mosquitoes were gently blew to the test tubes, these may have been excited by the odours of that blow and may have jumped the faster to find the source of these human odours.

That not irritated mosquitoes were not more resistant than the control lines and that resistance decreases irritancy suggests there are no genetic correlations between the two and this is in contradiction with what we expected. Indeed, because the genes of target site resistance confer nerve insensitivity to insecticides [42] we expected these to be also selected through the selection of mosquitoes that were staying the longer on the deltamethrin-impregnated paper, thus the ones that were the less irritated. Despite so, we discover that other genes than the one responsible of resistance are able to make mosquitoes less irritated. Thus, similarly to the insecticidal action of ITNs, the contact irritancy may select the mosquitoes that are able to stay longer on the treated net, thus increasing the number of mosquitoes that may be able to transmit their parasites despite the presence of ITNs. On the one hand, both the genes of resistance and the ones responsible of weak irritancy may thus reduce the personal protection offered by ITNs, on the other, they may help to maintain community protection as they make mosquitoes staying longer in contact with the insecticides and thus increases their chance of dying [29,44,45].

Although resistance seems to reduce the personal protection offered by ITNs, most of studies showed that the treated nets still prevent mosquitoes to transmit parasites like malaria for example [29,33,143,144]. However, we argue that to fully understand the impact of insecticide resistance on the efficacy of ITNs, we must consider whether mosquitoes are infected by malaria parasites or not. Indeed, with a laboratory setting, we recently showed that partially resistant *A. arabiensis* mosquitoes infected with *Plasmodium falciparum* sporozoites (the stages of the human malaria parasite that are infectious for human) were no more repelled by permethrin-treated nets [89]. Thus, it suggests that the combination of resistance and malaria infection in mosquitoes may drastically reduce the personal protection ITNs may offer.

Our observation about spatial repellency are a bit less straightforward than the one for irritancy and resistance. As expected from previous studies [reviewed in 71], the deltamethrin globally spatially repelled the mosquitoes. However, first, we found no difference in spatial repellency between all the selection-treatments and second, we were not able to select, at least during 10 generations, both the mosquitoes that were not spatially repelled or the ones that were strongly repelled. Because deltamethrin was not strongly spatially repellent, with only 1.2 more mosquitoes choosing the untreated paper in our experiment, we expected we would have needed more generations to select the genes of the few mosquitoes that were actually strongly or weakly spatially repelled.

Overall, we did not find any genetic correlations between contact irritancy and resistance in mosquitoes. While resistant mosquitoes were less irritated, the ones that were selected to be not irritated showed no higher resistance compared to the control lines. These results first confirmed that

by making mosquitoes less irritated, resistance decreases the personal protection offered by an ITNs. Second, they suggest that the selection of the mosquitoes that stay the longer on a treated net, thus the one that are able to pass or bite through it, may increase the number of mosquitoes that are able to transmit their parasites despite the presence of ITNs. If true, this might increase the chance for mosquito-borne parasites to be maintained in their environment. Globally, our results will therefore give new insights to understand the real epidemiological impact of ITNs and may help to develop or improve our insecticide-based control tools.

Chapter 5: Malaria infection in mosquitoes decreases the personal protection offered by permethrin-treated bed nets

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5.1 Abstract

Background: Insecticides targeting adult mosquitoes are the main way of controlling malaria. They work not only by killing mosquitoes, but also by repelling and irritating them. Indeed their repellent action gives valuable personal protection against biting mosquitoes. In the context of malaria control this personal protection is especially relevant when mosquitoes are infectious, whereas to protect the community we would prefer that the mosquitoes that are not yet infectious are killed (so, not repelled) by the insecticide. As the infectious stage of malaria parasites increases the motivation of mosquitoes to bite, we predicted that it would also change their behavioural response to insecticides.

Results: With two systems, a laboratory isolate of the rodent malaria *Plasmodium berghei* infecting *Anopheles gambiae* and several isolates of *P. falciparum* obtained from schoolchildren in Tanzania that infected *Anopheles arabiensis*, we found that mosquitoes harbouring the infectious stage (the sporozoites) of the parasite were less repelled by permethrin-treated nets than uninfected ones.

Conclusions: Our results suggest that, at least in the laboratory, malaria infection decreases the personal protection offered by insecticide-treated nets at the stage where the personal protection is most valuable. Further studies must investigate whether these results hold true in the field and whether the less effective personal protection can be balanced by increased community protection.

5.2 Background

Indoor residual spraying (IRS) and insecticide-treated bednets (ITNs) are among the most commonly used and cost-effective control measures against malaria [117]. Indeed, these measures significantly reduced malaria transmission, for example, in sub-Saharan Africa [118]. Both protect against malaria by repelling mosquitoes and by killing mosquitoes that rest on the treated surface after having taken a blood meal [4,38,71,141,145]. ITNs give additional protection by creating a physical barrier that reduces human-mosquito contacts.

The repellent action of IRS and ITNs offers effective personal protection [15–18] by reducing the number of bites (infectious bites in particular) on users; their insecticidal action offers protection to the community, first, by killing mosquitoes before the malaria parasite has completed its development and become transmissible and secondly, by killing infectious mosquitoes [38]. A striking example of community protection is that a 75% coverage of ITNs in a rural community in Tanzania decreased the entomological inoculation rate of the people that did not use an ITN by a factor of 18 [13]. To some extent, the two levels of protection oppose each other: strongly repellent nets, which give strong personal protection, let only few mosquitoes contact the insecticide on the nets, thus killing few mosquitoes and giving weak community protection [38–41].

To weaken this trade-off, we would want an ITN to repel mainly infectious mosquitoes, thus protecting users against being infected, but to repel other mosquitoes only weakly, thus maintaining community protection by lowering the number of mosquitoes that become infectious. Our prediction, however, was the opposite: as malaria changes the host-seeking behaviour of mosquitoes, its infectious stages would decrease repellency to give less rather than more personal protection against infection.

Indeed, infection with the infectious stage (sporozoites) increases the number of human-mosquito contacts in several ways. Sporozoite-infected mosquitoes have been found to be more attracted to human odours than uninfected ones [46,59], though not all experiments show the same pattern [49]. Sporozoites also increase the mosquito's persistence and biting rate [51–53,55] and increase the likelihood that the mosquitoes bite a second time to top up their blood meal [54]. These changes of behaviour probably evolved in order to increase the transmission of the parasite [57]. The general pattern of the behavioural changes - that sporozoite-infected mosquitoes are more motivated to bite than uninfected ones - may well have as a consequence that sporozoite-infected mosquitoes are less repelled by insecticides and therefore more likely to be killed by them.

The aim of this study was to test whether permethrin-treated bednets repel mosquitoes harbouring sporozoites less than uninfected mosquitoes (we chose to work with permethrin because it is one of

the most used pyrethroids for insecticide-treated nets [90]). To do so, we performed two experiments in the laboratory that differed in the malaria parasite, the mosquito, and the way we measured the repellency of permethrin. In a first experiment with the rodent malaria *Plasmodium berghei* infecting *Anopheles gambiae*, mosquitoes chose between feeding through a permethrin-treated net or through an untreated net. In a second experiment with the human malaria *P. falciparum* infecting *An. arabiensis*, mosquitoes had to pass through a treated or untreated net if they wanted to reach the blood source. Note that, while for simplicity we use the term repellency in both experiments, we measure different behaviours. In the first, we measure *preference* of untreated nets, whereas in the second we measure the *motivation* to bite despite the presence of the insecticide. Note also that in both experiments, repellency refers to a combination of spatial repellency and contact irritancy [71], for our experimental design does not let us distinguish between the two.

5.3 Methods

5.3.1 *Plasmodium berghei* and *Anopheles gambiae*

5.3.1.1 Mosquito and parasite

We used the Kisumu colony of *An. gambiae* originating from western Kenya [91] and the mouse malaria *P. berghei* ANKA, modified to express the green fluorescent protein (GFP). Infected mice harbouring gametocytes were obtained from Volker Heussler (Institute of Cell Biology, University of Bern) and used to feed and infect the mosquitoes.

5.3.1.2 Mosquito rearing and infection

We haphazardly selected larvae from the colony and reared them individually at 26 ± 1 °C in 12-well plates with a standard level of fish food (Tetramine) [91]: day of hatching of eggs, 0.04 mg per larva; 1 day after hatching, 0.06 mg; day 2, 0.08 mg; day 3, 0.16 mg; day 4, 0.32 mg; day 5 and later, 0.6 mg. Upon pupation, each mosquito was moved to a 120 ml plastic cup.

Three to four days after emergence, we moved the mosquitoes to an insectary maintained at 19 ± 1 °C and 70 ± 5 % of humidity for an efficient development of *P. berghei* in mosquitoes [70]. We haphazardly let females feed for 15 min on an infected or an uninfected mouse that had been anaesthetized. To replicate the infection-treatment, we used two infected and two uninfected mice, and let 100 mosquitoes feed on each one. Twenty-four hours after feeding, engorged mosquitoes were placed into 120 ml individual plastic cups; unfed mosquitoes were discarded. We gave the mosquitoes access to a cotton ball soaked with a 10% sugar solution up to 24 h before the behavioural test.

5.3.1.3 Measuring repellency

We defined repellency of a permethrin-treated net as the preference of mosquitoes for an untreated net when given the choice of feeding on a blood source through an untreated net or a permethrin-treated one. We measured the preference with a two-way choice apparatus (Fig. 1), where mosquitoes could choose to fly towards and attempt to bite an arm held onto a cage containing either a 30 × 30 cm piece of net with permethrin (270 mg/m^2) or netting without permethrin (Trek 1 or Trek, Katadyn France, Grenoble, France). A ventilator guided the odours (at a speed of about 20 cm/s) from the two cages into a central cage, into which the mosquitoes had been placed. The side of the insecticide was alternated among tests so that any side preference was avoided. Moreover, the potential bias for one of the sides of our setup was controlled by testing shortly before our experiment that there was no preference for either side when uninfected mosquitoes were given the choice of two untreated net. In eight replicates (each with 10 uninfected mosquitoes aged 20 to 22 days) a repeated G-test of goodness-of-fit [146] showed strong support for no preference [total-G = 1.48, *df* = 8, *P* = 0.99, pooled-G = 0.385, *df* = 1, *P* = 0.53, with no heterogeneity among the replicates (*G* = 1.1, *df* = 7, *P* = 0.99)].

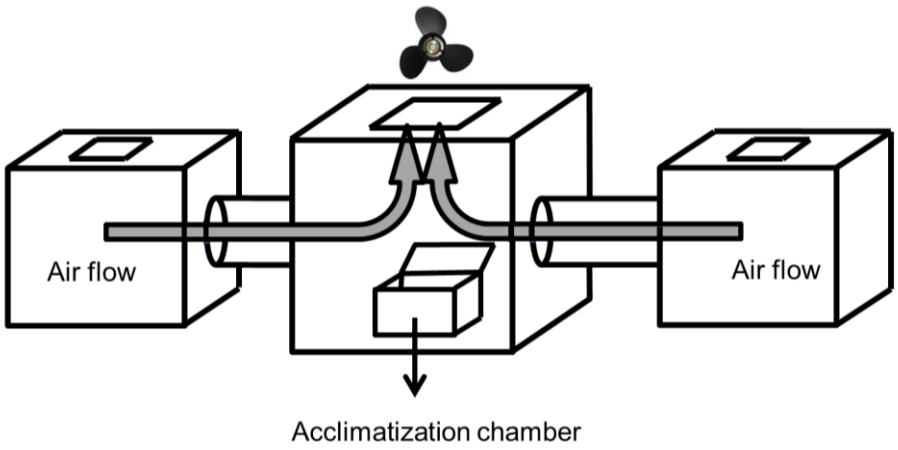

Figure 1: Two-way choice apparatus to test the effects of malaria infection on insecticide repulsion in the experiment with P. berghei infecting An. gambiae. The central cage (25 × 25 × 25 cm) was connected to two test cages (20 × 20 × 20 cm) with 15 cm length tubes (diameter 10 cm). The two small cages had a hole covered by the permethrin-treated net or the untreated net on the top. Mosquitoes were placed in the 15 × 10 × 5 cm acclimatization chamber for 2 min before their behaviour was tested. The arrows represent the direction of the air flow

Twenty to twenty-two days after the blood meal we tested 90 uninfected mosquitoes and 80 infected ones in groups of 10. For each replicate, we placed the 10 mosquitoes into the central acclimation chamber and KT put his arms as attractants onto the nets of the two small cages. After letting the mosquitoes acclimatize for 2 min we opened the chamber and gave the mosquitoes 8 min to make a choice. We recorded the number of mosquitoes in each cage and put the mosquitoes into a freezer (-20 °C) until further analysis.

5.3.1.4 Malaria infection

DNA was extracted with DNAzol (MRC inc., Cinncinati, OH, USA) according to the manufacturer's instructions modified by Rider et al. [147]. The head and thorax of mosquitoes were smashed with a pellet in 200 µl of DNAzol. The solution was incubated for 20 min at 55 °C and then centrifuged at 20,000× *g* for 10 min. One hundred and seventy microliters of the supernatant were transferred to a tube together with 3 µl of PolyAcryl Carrier (MRC inc.) and 100 µl of cold (-20 °C) 100% ethanol. After centrifuging at 15,000× *g* for 8 min we discarded the ethanol. We then washed the DNA by adding 0.8 ml of cold 75% ethanol and centrifuging the tube for 5 min at 15,000× *g*. Ethanol was again discarded and the pellet of DNA was dried in a Speedvac for 15 min at 45 °C. Finally, the DNA was eluted in 50 µl of autoclaved, deionised water.

Sporozoite infection was confirmed by PCR using a modified protocol established by Rider et al. [147]. PCR amplification was done with the T3000 thermocycler [Analytik Jena AG (formerly Biometra), Jena, Germany] with 3 µl of DNA and 1 µl of a 10 µM solution of each primer, forward (5'-ACG ATG ATA TAG ATC AAA T-3') and reverse (5'-TAC CTA AGC TTC TTG CGT A-3'), which amplify a 111 bp sequence of the merozoite surface protein-1 (*MSP-1*) gene of *Plasmodium berghei* NK65 and ANKA. DNA was amplified during 40 cycles with denaturation at 95 °C, annealing at 54 °C and extension at 72 °C, each for 45 s. Infection was confirmed from the correct band of DNA after electrophoresis on a 1.8% agarose gel stained with the RedSafeTM Nucleic Acid stain (iNtRON Biotechnology, Seongnam-si, South Korea).

5.3.1.5 Mosquito body size

Wing length was taken as a measure of mosquito body size. Wings were placed onto a glass slide and scanned. We used the software ImageJ to measure the distance from the distal end of the alula to the tip of the wing (vein R3) without the fringes [148,149].

5.3.1.6 Statistical analysis

We analysed the proportion of mosquitoes that responded with a generalized linear mixed model (GLMM) with a binomial distribution where the infection status was set as a nominal factor and the replicate as a random factor. Using only the mosquitoes that responded, we analysed the preference with a GLMM, where the choice of mosquitoes was set as the response variable, the infection status as a nominal factor, the wing length and the day of test (i.e. the mosquito's age) as covariates, and the replicate as a random factor. We excluded exposed but not infected mosquitoes from the analyses, as there were too few (n = 7) that responded during the test to enable any meaningful statistical analysis or interpretation. The significance of the effects was determined with the ANOVA function (type II)

from the "*car*" package [97]. The analysis was performed with the software R [98] and the Rstudio interface [99]. For the mixed effect models, we used the package "*lme4*" [100].

5.3.2 *Plasmodium falciparum* and *Anopheles arabiensis*

5.3.2.1 Study site and patients

The study was conducted from the beginning of May to the end of July 2016 near Bagomoyo in the coastal region of Tanzania, where *P. falciparum* is the main parasite causing malaria. All laboratory-based work was done at the Kingani insectary site of the Bagamoyo Research and Training Unit (a branch of the Ifakara Health Institute). Malaria parasites were obtained from 6 to 14 year-old children from villages around Bagomoyo. A first blood screening was done at the schools of the villages. We used malarial rapid diagnosis test (SD BIOLINE Malaria Ag P.f/Pan, Standard Diagnostic, Gyeonggi-do, South Korea) to determine if children were infected or not. Children carrying both *P. falciparum* and another *Plasmodium* species were treated with antimalarial drugs (Coartem©, Novartis, Basel, Switzerland) according to the national guidelines. Blood smears of children infected only with *P. falciparum* were coloured with 10% Giemsa and checked under a microscope (oil film 100× magnification) for the presence of gametocytes. Children that harboured only the asexual form of *P. falciparum* were treated with antimalarial drugs (Coartem©, Novartis) at school. Children that harboured gametocytes were driven together with their parents or their teacher to the laboratory of Kingani, where we took a sample of 3 ml of blood to infect mosquitoes (see below). They were then driven back to their villages and the children received an antimalarial treatment.

5.3.2.2 Mosquitoes

We used the mosquito *An. arabiensis* from a partially permethrin-resistant colony established in 2006 in Sakamaganga, Kilombero, Tanzania and maintained in an insectary at the Bagamoyo Research and Training Unit. Mosquitoes were maintained at a temperature of 27 ± 2 °C with a relative humidity of 70–80% and 12:12 h of dark:light photocycle. Larvae were reared in groups of 250 mosquitoes in trays containing 1 l of water and given the quantities of Tetramine fish food mentioned above. Adults were maintained in 35 × 35 × 35 cm cages and allowed to feed on a 10% glucose solution. Males were removed from the cages every 24 h to avoid fertilization. Twenty-four hours before the infection, female mosquitoes were starved and placed in groups of 50 into paper cups.

5.3.2.3 Blood preparation and infection of mosquitoes

Mosquitoes were fed on the blood of 5 children containing gametocytes of the human malaria parasite, *Plasmodium falciparum*, obtained as described above. The blood was prepared according to the procedure described by Bousema et al. [150]. The blood was centrifuged for 2 min at 3000× *g*. The serum was removed and replaced with a European AB serum, so that transmission-blocking factors were avoided [151]. Half of the blood of each child was maintained at 37 °C; the other half was heated to 43 °C for 15 min to kill the malaria parasites, preventing infection of mosquitoes without changing other blood characteristics [152,153]. Mosquitoes were then allowed to feed in the dark for 45 min from micro-feeders containing 300 µl of infected or heat-killed blood maintained at 37 °C. Twenty-four hours after infection, unfed mosquitoes were removed. The fed ones were placed in groups of 20 to 25 individuals into new cups and maintained in a 2.5 security laboratory at 25–27 °C and 70–80% humidity, with 12:12 h of dark:light photocycle. They were allowed to feed on a piece of cotton soaked in a 10% glucose solution until 24 h before the behavioural tests. The tests were done 14 days after feeding, enabling the malaria parasite to produce sporozoites.

5.3.2.4 Measuring repellency and 24 h mortality

In this experiment we assessed whether the attraction of a host to a hungry (unfed) mosquito can overcome the repellency by the permethrin. We measured the repellency of the bednets to infectious or uninfected mosquitoes with a modified WHO tunnel (see Fig. 2). Mosquitoes were placed according to infection status and isolate in groups of 20 into one chamber (the release chamber) of the tunnel. KT placed his foot into the chamber on the opposite side (the stimulus chamber) to attract mosquitoes. His foot was protected from being bitten by a cloth with 150 µm mesh, which enables volatiles but not mosquitoes to pass through. The central chamber was separated from the stimulus chamber by a commercially available permethrin-treated mosquito net (Olyset net, Sumitomo Chemical Co. Ltd., Tokyo, Japan) or by an untreated mosquito net. The nets contained 7 holes (0.8 cm diameter), so that mosquitoes could pass through. The tunnel was covered with black tissue, for *An. arabiensis* prefers to bite during the night. We left mosquitoes in the tunnel for 20 min and then recorded the number of mosquitoes in each part of the tunnel. They were considered as unresponsive, if they remained in the release chamber; responsive but repelled if they were found in the central chamber; and responsive and not repelled if they were found behind the net in the stimulus chamber. After the test, mosquitoes were placed in paper cups for 24 h with access to 10% glucose solution and their 24 h mortality was recorded. They were then placed into Eppendorf tubes containing silica gel for transport to the University of Neuchâtel for further analysis.

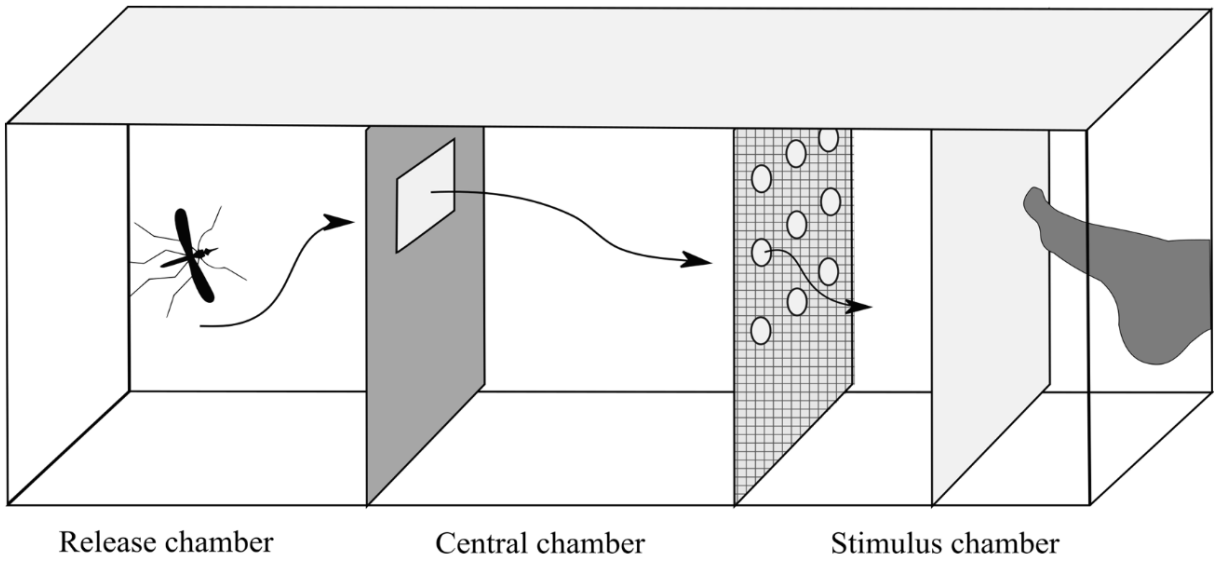

Figure 2: Modified WHO tunnel to test the repellency of ITNs during the experiment with P. falciparum infecting An. arabiensis. Mosquitoes were placed in the released chamber and allowed to fly in the direction of the stimulus chamber where KT put one of his feet (protected by chiffon with 150 µm mesh). They had to pass through an opening made with cardboard with a rectangular hole of 8 × 12 cm to be considered as responsive, and then could pass through a net with holes to approach KT's foot if they were not repelled. The arrows represent an example route for mosquitoes to go to the stimulus chamber

5.3.2.5 Malaria infection

Sporozoite-infection of mosquitoes was confirmed by PCR using the protocol established by Padley et al. [154]. DNA extraction of the head and thorax of mosquitoes was performed with DNAzol according to the procedure described above.

PCR was performed with the T3000 thermocycler (Analytik Jena AG). Forward (5'-AAC AGA CGG GTA GTC ATG ATT GAG-3') and reverse primers (5'-GTA TCT GAT CGT CTT CAC TCC C-3') amplified a 276 bp sequence of *Plasmodium falciparum*. PCR was done on 2 µl of DNA with 240 nM of each primer for a final volume of 25 µl. DNA was amplified during 40 cycles with denaturation at 95 °C, annealing at 60 °C and extension at 72 °C, each for 45 s. Infection was confirmed from the correct band of DNA by electrophoresis on a 1.8% agarose gel stained with the RedSafeTM Nucleic Acid stain (iNtRON Biotechnology).

PCR amplifications were also done on the abdomens of mosquitoes that were exposed to infected blood in order to detect oocyst infection. We followed the same procedure for the PCR and the DNA extraction as for sporozoite-infection, except that we added only 150 µl of DNAzol for each abdomen.

5.3.2.6 Mosquito body size

The wing length was measured as the distance from the distal end of the alula to the tip of the wing (vein R3) without the fringes.

5.3.2.7 Statistical analysis

We estimated repellency in two ways: (i) as the proportion of mosquitoes that did not respond to the human cue, i.e. that stayed inside the releases chamber (Fig. 2); and (ii) as the proportion of responding mosquitoes that did not pass the net to move from the central to the stimulus chamber. The former is an expression of spatial repellency, the latter could be due to a combination of spatial repellency and contact irritancy. We analysed the likelihood to respond to human cues with a GLMM where the type of net and the infection status [unexposed (fed on blood containing heat-killed parasites); exposed but not infected; or infectious] were nominal factors, wing length was a covariate and the isolate (the child from whom the parasite had been obtained) and the replicate (the group of 20 mosquitoes tested simultaneously) were random factors. We analysed the likelihood that responding mosquitoes passed through the net with a GLMM that included the type of net and the infection status as nominal factors, wing length as a covariate, and the isolate and the replicate as random factors. Mosquitoes that were infected, but harboured only oocysts, were removed from the analyses. We further analysed to variations of this basic model. First, as the true infection status of 'exposed but uninfected' mosquitoes was not clear, we left them out in a second analysis; this did not change the qualitative results. Secondly, we compared the repellency of the treated and untreated nets separately.

Mosquito mortality 24 h after the test of repellency was also analysed with a GLMM. The model included whether mosquitoes died or not as the response variable, and the same factors and covariate as the analysis for repellency. We also compared the mortality of each net individually.

All analyses were performed with the software R [98] and the Rstudio© interface (version 1.0.143) [99]. For the mixed effect models (which were done with the package "*lme4*" [100]), the statistical significance of each factor and covariate was determined with a type 3 ANOVA from the package "*car*" [97].

5.4 Results

5.4.1 *Plasmodium berghei* and *Anopheles gambiae*

Of the 80 mosquitoes exposed to gametocytic mice, 35 did not harbour sporozoites and were therefore excluded from the analysis. The percentage of mosquitoes responding to human cues was 55.6% (95%

confidence interval, CI: 45.3–65.4%) if they were uninfected and 66.7% (95% CI: 52.1–78.6%) if they harboured sporozoites. This difference was not statistically significant (χ^2 = 1.52, *df* = 1, *P* = 0.22).

Including only the mosquitoes that responded let us analyse the choice of 80 mosquitoes (30 infected and 50 uninfected) when facing a permethrin-treated or a control net. While 68% (95% CI: 54.2–79.2%) of the uninfected mosquitoes avoided the cage with the insecticide, only 43.3% (95% CI: 27.4–60.8%) of the infected ones did (χ^2 = 4.11, *df* = 1, *P* = 0.043) (Fig. 3). Neither the mosquito's wing length (χ^2 = 0.46, *df* = 1, *P* = 0.5) nor its age (χ^2 = 0.06, *df* = 1, *P* = 0.81) affected the choice of mosquitoes. There was also no effect of the interaction between mosquito's age and their infection status (χ^2 = 0.04, *df* = 1, *P* = 0.85).

Figure 3: Proportion of repelled mosquitoes as a function of their infection status. Mosquitoes were considered as repelled if they were found in the cage without the permethrin-treated net. The bars represent the 95% confidence interval

5.4.2 *Plasmodium falciparum* and *Anopheles arabiensis*

Of the mosquitoes that survived up to 14 days after blood-feeding, we selected haphazardly 80 mosquitoes from each of 3 isolates, 160 from a fourth one and 360 from a fifth (these numbers reflect the greater survival for the latter two isolates). We tested 50 mosquitoes that had fed on blood with heat-killed parasites; none of them were infected. Of the mosquitoes that had fed on infectious blood, 29.0% harboured sporozoites (half of these also harboured oocysts; in our analyses we did not distinguish between the mosquitoes with or without oocysts, both groups were considered as infectious). A further 6.3% harboured oocysts, but had no sporozoites; we removed these from the analysis.

The percentage of mosquitoes that responded to host cues (that is, that moved out of the release chamber) was 83.7% (95% CI: 79.6–87.1%) for the unexposed ones (those fed on blood containing heat-killed parasites), 79.3% (95% CI: 73.8–83.9%) for exposed-but-uninfected mosquitoes and 89.1% (95% CI: 81.9–93.7%) for the infectious ones, (χ^2 = 7.4, *df* = 2, *P* = 0.02). The difference of the

percentage of responders between the untreated and treated nets was less than 2% for each infection status (main effect of net: $\chi^2 = 0.01$, $df = 1$, $P = 0.92$; interaction between infections status and net: $\chi^2 = 0.13$, $df = 2$, $P = 0.94$). Wing length had no effect on the proportion of responders ($\chi^2 = 1.98$, $df = 2$, $P = 0.16$).

The analysis comparing the proportion of mosquitoes passing the net into the stimulus chamber included 611 responding mosquitoes, with 286 mosquitoes (151 not exposed, 89 exposed-but-uninfected and 46 infectious) that were tested on an untreated net and 325 (167 not exposed, 106 exposed-but-uninfected and 52 infectious) mosquitoes that were tested on the permethrin-treated Olyset net. About of the quarter of the responding mosquitoes remained in the central chamber, if they were tested with an untreated net. This proportion was not affected by infection status: unexposed: 25.2% (95% CI: 18.9–32.9%.); exposed-but-uninfected: 25.8% (95% CI: 17.9–35.8%); infectious: 26.1% (95% CI: 15.6–40.3%) ($\chi^2 = 0.05$, $df = 2$, $P = 0.97$). When tested on an Olyset net, in average 40.6% of mosquitoes were repelled, with the response ranging from 46.7% (95% CI: 39.3–54.3%) of the unexposed mosquitoes down to 23.1% (95% CI: 13.7–36.1%) of the infectious mosquitoes ($\chi^2 = 7.67$, $df = 2$, $P = 0.02$). Pairwise comparisons showed that unexposed mosquitoes were repelled more than sporozoite-infected ones ($z = 2.71$, $P = 0.007$), and that exposed-but-uninfected mosquitoes were not significantly different from either unexposed ($z = 1.23$, $P = 0.22$) or infectious ones ($z = 1.74$, $P = 0.08$).

The full analysis, pooling the data with the two nets, confirmed that the mosquitoes were less likely to pass through the Olyset than the untreated net ($\chi^2 = 16.06$, $df = 1$, $P < 0.001$), and that the effect of the net depended on the infection status. While the interaction between net type and infection was not quite significant when we compare all three infection groups ($\chi^2 = 4.66$, $df = 2$, $P = 0.098$), it was significant when we omitted the exposed-but-uninfected mosquitoes ($\chi^2 = 4.44$, $df = 1$, $P = 0.035$). Wing length did not affect the behavioural response in either of these analyses (three infection groups: $\chi^2 = 0.02$, $df = 1$, $P = 0.90$; unexposed *vs* infectious $\chi^2 = 0.45$, $df = 1$, $P = 0.51$).

Overall, uninfected mosquitoes were more repelled by the Olyset than the untreated net, but infectious mosquitoes were just as likely to pass through the Olyset as the untreated net (Fig. 4a).

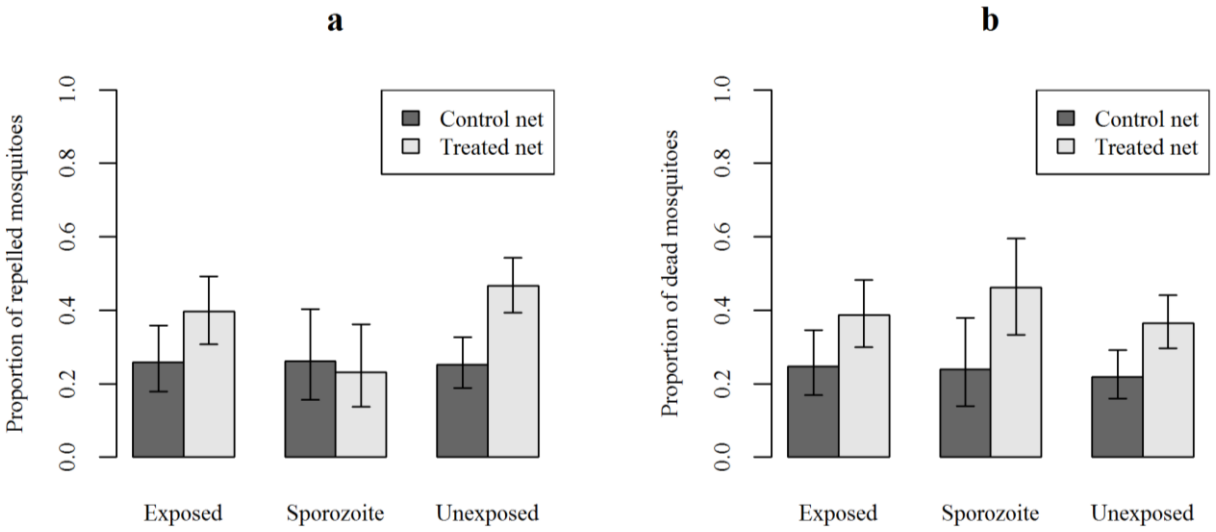

*Figure 4: **a** Proportion of mosquitoes found in the central cage (and therefore responded to the odour but were repelled by the net) for the two types of net [Olyset permethrin-treated net (light grey) or control untreated net (dark grey)] and for the three infection statuses. **b** Proportion of mosquitoes that died within 24 h after the behavioural test, as a function of the type of net and infection status. These proportions represent the overall mortality, combining the mortality of the mosquitoes considered as repelled and the one considered as unrepelled. For both graphs the bars represent the 95% confidence interval*

Twenty-four hours after the test with the untreated net 23.1% of mosquitoes had died, independently of their infection status: unexposed 21.9% (95% CI: 16–29.1%); exposed-but-uninfected: 24.7% (95% CI: 16.9–34.6%); infectious: 23.9% (95% CI: 13.9–37.9%) (χ^2 = 0.55, *df* = 2, *P* = 0.76). After the test with a permethrin-treated net, 38.8% of mosquitoes had died, again independently of their infection status unexposed: 36.5% (95% CI: 29.6–44.1%); exposed-but-uninfected: 38.7% (95% CI: 30–48.2%); infectious: 46.2% (95% CI: 33.3–59.5%) (χ^2 = 1.45, *df* = 2, *P* = 0.48).

In the analysis pooling the two types of net, we found that the Olyset net increased the risk of dying by a factor of 1.7 (χ^2 = 15.05, *df* = 1, *P* < 0.001, Fig. 4b) and we confirmed that there was no significant effect of the infection status (χ^2 = 1.48, *df* = 2, *P* = 0.48) and that neither the wing length (χ^2 = 0.0002, *df* = 1, *P* = 0.99) nor the interaction between the type of net and the infection status (χ^2 = 0.46, *df* = 2, *P* = 0.79) affected the mosquito's mortality.

5.5 Discussion

Our results with two experimental systems show that infection by malaria affects the behavioural response of mosquitoes to insecticides. In particular, in both systems, infection by sporozoites negated the repellency of permethrin, therefore decreasing the personal protection offered by an ITN.

The decrease of repellency was not only observed with two combinations of malaria and mosquito species, but also with two ways of measuring the repellent effect of an insecticide. In the first

experiment, when given the choice between flying into (and biting in) a chamber containing either an untreated or a treated net, uninfected mosquitoes preferred to fly into the chamber that was not protected by permethrin (showing that they were repelled by the insecticide), while infectious mosquitoes showed no preference. In the second, mosquitoes were not given the choice of an insecticide-free chamber, but we measured the likelihood that mosquitoes would pass an untreated or a treated net to get to their blood meal. Again, uninfected mosquitoes were repelled, and thus less likely to fly through a treated bednet than a treated one to reach their source of blood, while infectious mosquitoes responded similarly to treated and untreated nets.

Both of these aspects of repellency could be a consequence of spatial repellency (which impede mosquitoes from approaching treated nets) or contact irritancy (which makes mosquitoes fly away once they contact a treated net). However, our second experiment gives some evidence that spatial repellency is not an important factor. If it were important, we would have expected that fewer mosquitoes move towards a treated net (and thus stay in the release chamber) than towards an untreated net. The proportions, however, did not differ. This corroborated the patterns observed in several other studies that found no spatial repellency, but strong irritancy for permethrin (reviewed in [71]).

The proportion of mosquitoes remaining in the release chamber did, however, depend on their infection: sporozoite-infected mosquitoes were more likely to move out of the chamber and towards the human cues than uninfected or exposed-but-uninfected mosquitoes. This is in line with previously observed changes of mosquitoes' biting behaviour induced by malaria, in particular that sporozoite-infection increases the host attraction [47] and the motivation and persistence of mosquitoes to obtain a blood meal [51–55] by enhancing the responsiveness of mosquito's olfactory receptors [47].

The possible mechanism of the decreased repellency of infectious mosquitoes by permethrin may be based on this behavioural manipulation: infectious mosquitoes could be more motivated to pass the permethrin-treated net to obtain their blood meal than uninfected ones. Alternatively, the chronic infection by malaria may simply damage in some way the receptors (or their integrative pathways) of the mosquitoes, analogously to the effect of a previous exposure to an insecticide in the odour receptors of *Aedes aegypti* [155]. Thus the mosquitoes would be less aware of the insecticide's odour or irritancy, and therefore less repelled by it. Indeed, malaria infection reduces the levels of several proteins in the head of mosquitoes, including a synaptic and a neural signaling protein, though the functions of these proteins are not known [156].

Our observations on mortality are a bit less straightforward than those on behavior. As expected, the permethrin-treated net killed more mosquitoes than the untreated net. However, although the sporozoites increased the mortality from about 37% to about 46%, this difference was far from statistically significant. This contrasts our expectation that the increased contact of infectious mosquitoes with the insecticide would increase the likelihood that they are killed. A likely explanation for the lack of statistical significance is simply a lack of power. If (as the numbers of our experiment indicate) about 20% fewer mosquitoes are repelled and these are 10% more likely to be killed, we would need a large sample size to detect the combined effect as a statistically significant result. Alternatively, infectious mosquitoes may pass through the net so quickly that their exposure is too short to kill them. It would be important to know which of these (or any other) explanation is correct, for community protection and the rate of the evolution of insecticide resistance both depend mainly on the insecticidal property of ITNs [38]. Furthermore, to fully determine the level of community protection offer by ITNs it would be important to take in account the weaker attraction or motivation of oocyst-infected mosquitoes [47,54]. Indeed, it might increase the overall number of infectious mosquitoes, for oocyst infection might decrease the mosquito's risk of dying due to ITNs or host defence behaviours [54,157].

A caveat of our study is that both experiments were done in laboratory conditions, which can differ considerably from natural situations. In particular, laboratory infections might differ from natural infection and thus might influence the density of the sporozoites in the mosquitoes' salivary glands [158–160], which in turn can influence the malaria-induced change of behaviour [57]. Nevertheless, we suggest that malaria infection in mosquitoes can decrease the repellency of insecticides and may reduce the personal protection at the stage when the protection is most relevant.

5.6 Conclusion

Insecticide-based tools, like ITNs and IRS, have proven their efficacy against malaria [7,118] and remain the most cost-effective tools for malaria control [117]. However, to fully understand and predict the impact of ITNs on the epidemiology of malaria, we need to know how malaria affects the personal and community protection offered by the nets. Our main finding is that the malaria infection, at least in the laboratory, reduces the personal protection offered by insecticides. We suggest that further studies should investigate whether our laboratory results are consistent in the field and go on to investigate whether the less effective personal protection observed here can be balanced by increased community protection.

5.7 Ethic approval and consent to participate

All procedures that involved mice were conducted in accordance with the Swiss Tierschutzgesetz guidelines (TSchG; Animal Rights Laws). They were approved by the ethical committee of the University of Bern (Permit Number: BE109/13). Mice were anesthetized with an intra-peritoneal injection of 8.5 ml/kg of a mix of Xylazine Xylasol® (solution: 20mg/ml), Ketamine Ketasol ® (solution: 100 mg/ml) and PBS [92]. Obtaining and using the blood of the Tanzanian schoolchildren was approved by the Ethical Committee of the University of Neuchâtel, the Ifakara Health Research and Development Centre, the Medical Research Coordination Committee (MRCC) of the National Institute of Medical Research (NIMR) and the Tanzanian Commission for Science and Technology (COSTECH). Parents were invited to attend a sensitization meeting at the school premises. The children as well as their parents and teachers were informed about the project, and those who were interested to participate were given an informed consent form. The parents had to read and sign the form in order to allow us to enroll their children in the study.

5.8 Funding

Financial support for the field experiment in Tanzania was provided by the Fonds des Donations from the University of Neuchâtel, the Fondation Hans Wilsdorf, the Schmidheiny foundation and the Rotary Club Genève Groupe SERVIR (Marc Plojoux).

Chapter 6: Parasite load affects mosquito's salivary apyrase activity in a rodent malaria model

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6.1 Abstract

Mosquitoes infected by sporozoites, the infectious stage of malaria, bite more frequently than uninfected mosquitoes. One of the mechanisms underlying this behavioural change appears to be that the sporozoites reduce the activity of apyrase, an ADP-degrading enzyme that help the mosquitoes to locate blood. Using the parasite *Plasmodium berghei* and the mosquito *Anopheles gambiae*, we confirmed that sporozoite infection alters the host-seeking behaviour of mosquitoes by making them more likely to refeed after a first blood meal, and that apyrase activity is one of the mechanisms of the increased biting persistence and motivation of infectious mosquitoes. We showed that apyrase activity decreases as the sporozoite load increases, and that mosquitoes with lower apyrase activity take up less blood (making it more likely that they would return to top up their blood meal). Our results give new insights in understanding how malaria parasites change their hosts to affect their own transmission.

6.2 Introduction

Malaria parasites change the behaviour of mosquitoes in several ways that are likely to increase their transmission [57–59,161]. When mosquitoes harbour sporozoites (the stage that is infectious to humans), they are more attracted to human [46–48] (even if not all studies show the same pattern [49]), they are more persistent [52,53], probe for blood longer [50] and have a greater biting rate [51]. They are also more likely to refeed after a partial or a complete blood-meal [54,162], and they bite more people [55]. In contrast, oocysts (the stage in the mosquito that is not infectious) decrease the biting motivation of the mosquito [47,53,54] presumably decreasing the mosquito's biting-associated mortality [64] and increasing their own chance of surviving to become infectious. Additionally, humans carrying gametocytes (the stage that is infectious to mosquitoes) are more attractive to mosquitoes [163,164].

Some of the mechanisms of the behavioural manipulation by malaria parasites are starting to be understood. In a human infected with *Plasmodium falciparum* gametocytes, for example, the parasite changes the composition and increases the emission of volatiles, making the person more attractive to mosquitoes [165]. The mechanisms underlying behavioural changes in sporozoite-positive mosquitoes are less clear. The potential changes in biting behaviour may originate from changes in the mosquito's saliva, which helps them to locate blood vessels [166]. This is in particular due to apyrase, an ADP-degrading enzyme that co-occurs with the sporozoites in the mosquito's salivary glands [167]. Apyrase activity is inversely related to the probing time of at least three anopheline species [168] and apyrase impedes coagulation [169]. As apyrase activity is reduced in the salivary glands of *Aedes aegypti* infected with sporozoites of *Plasmodium gallinaceum* [50], infectious mosquitoes will be able to take up less blood during the limited time of their contact with a person, which is likely to increase their biting rate and the probability that they bite several hosts [51].

While we thus have some ideas about the relationships between infection by sporozoites, apyrase activity and biting behaviour, there are no studies that consider these relationships explicitly. The main aim of our study was therefore to investigate with the mosquito *Anopheles gambiae* and the malaria parasite *Plasmodium berghei* the link between sporozoite load, apyrase activity and blood-feeding behaviour. We quantified sporozoite load and apyrase activity, and assessed their effects on some of the behavioural traits that were changed by the parasite in our earlier studies: the quantity of blood taken up during a given amount of time, the proportion of mosquitoes attempting to bite a second time a few hours after their first blood-meal, and the time they need to start probing during their second biting attempt [54]. A second aim was to test whether the sensitivity of mosquitoes to be

manipulated by sporozoites has a genetic basis. This is, to our knowledge, the first study to consider the possibility that different genotypes of mosquitoes are manipulated differently.

6.3 Methods

6.3.1 Mosquitoes and parasite

We used a Kisumu strain of the mosquito *Anopheles gambiae,* which originated in western Kenya [91], and we used a strain of *P. berghei* ANKA expressing the green fluorescent protein (GFP). To infect the mosquitoes, we obtained mice infected with gametocytes of the parasite from Professor Heussler at the University of Bern.

6.3.2 Mosquito rearing

We ran our experiment with eight iso-female families of mosquitoes. We first reared 100 larvae individually in 12 well-plates with the standard concentration of Tetramin Baby^{TM} fish food used in our lab (day of hatching of eggs: 0.04 mg, 1 day after hatching: 0.06 mg, day 2: 0.08 mg, day 3: 0.16 mg, day 4: 0.32 mg, day 5 and later: 0.6 mg). When they emerged, we haphazardly blood-fed 30 females, moved them to 120 mL individual cups and let them lay eggs. The larvae of 16 haphazardly selected females were then reared individually in 12 well-plates with the same food-regime as their mothers. Pupae were placed by family in 25x25x25 cm cages, and the females of each family were blood-fed 3 to 5 days after their emergence.

To run our tests, we haphazardly selected the larvae of 8 of these families and reared 3072 larvae individually in 12-well-plates with the same food-regime as above. Pupae were put individually in 50 mL falcon for emergence. Adult females were maintained in the falcon tubes and in an insectary at 26±1 C°, with 70±5 % of humidity with a 12:12 hours light cycle and had access to a piece of cotton soaked in a 10% solution of sugar. One day before infection, mosquitoes were starved and we gradually decreased the temperature to 19±1 C°, which is the optimal temperature for *P. berghei* development in mosquitoes [70].

Note that our families are thus the second generation of eight iso-female lines.

6.3.3 Infection

To obtain infected and uninfected mosquitoes, we fed 3 to 4 day old females on gametocytic or uninfected mice. Twenty mosquitoes of each family were fed on each of 4 infected and uninfected

mice, giving 80 exposed and 80 unexposed mosquitoes. We based this sample size on previous experiments, in which about 75% of the mosquitoes blood-fed on the mice, of which 80% were infected, of which 50% survived to the age they were tested. This would give about 25 infectious mosquitoes to test per family.

One day before infection, each group of 20 mosquitoes was placed into a 120 ml plastic cup covered by mosquito netting. On the day of infection, the mice were anaesthetized with a mix of Xylazine Xylasol® (solution: 20 mg/ml), Ketamine Ketasol ® (solution: 100 mg/ml) and PBS during this procedure. We injected 8.5 ml/kg of the mix by intra-peritoneal injection [92]. After anaesthesia, mice were placed onto the cups for 15 minutes. Twenty-four hours after blood-feeding, the engorged females were placed individually into 120 ml cups, and we gave them the possibility to feed on a piece of cotton soaked in a 10% solution of sugar.

6.3.4 Tests for behavioural changes

The procedure to measure the behaviour was adapted from a previous experiment [54]. Between 20 and 22 days after the blood-feeding (which is the time necessary to find sporozoites in the salivary glands of mosquitoes), females were given the opportunity to blood-feed on Kevin Thiévent's (KT) arms for 1.5, 3 or 4.5 minutes. About 2 hours after this first blood meal, we gave the mosquitoes the possibility to re-feed on KT's arms. In order to avoid any further uptake of blood by mosquitoes, they were removed from the arms as soon as they started to probe and the time to probe was recorded. If they did not attempt to probe within 4 minutes, we stopped the test. After the second test, mosquitoes were placed on ice for further dissection.

6.3.5 Dissection of salivary glands

Each pair of salivary glands was dissected in 0.15 M NaCl and placed in 1.5 ml Eppendorf containing 10 µl of Triton X-100 0.05%. We placed this salivary homogenate into the freezer at -20 C° for further analysis. 1 µl of that homogenate was used to evaluate the apyrase activity; the remaining 9 µl were used to quantify the sporozoite load.

6.3.6 Sporozoite load

To measure the sporozoite load in the mosquito's salivary glands, we used a modified protocol for a Real-Time PCR (RT-PCR), which allows to measure the number of *P. berghei* sporozoites in salivary glands of *A. gambiae* [147].

DNA extraction of mosquito salivary glands was obtained with a slightly modified DNAzol© procedure [89]. 125 µl DNAzol© were added to the 9 µl of salivary homogenate, then the mix was vortexed and incubated for 20 minutes at 55 C°. The solution was centrifuged at 20'000 g for 10 min. 170 µl of the supernatant was transferred in a new Eppendorf tube, to which we added 2 µl PolyAcryl Carrier (MRC inc.) and 100 µl cold (-20 °C) 100% ethanol for DNA precipitation. The solution was mixed by inversion and incubated for 5 minutes at room temperature. After a centrifugation at 15'000 g for 8 min, the ethanol was discarded. DNA was washed by adding 800 µl 70% ice ethanol and centrifuged at 15'000 g for 5 min. Ethanol was again discarded and DNA was dried in a speed-vac at 45 C° for 10 minutes. The final dried DNA was eluted in a volume of 40 µl distilled water and stored at -20 C° for further quantitative PCR.

The procedure for the RT-PCR was adapted from the protocol of Rider et al. [147]. Amplification was done with the LightCycler 96 ® system (Roche, Switzerland) with 3 µl DNA, 4 µl HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis Biodyne, Estonia) and 0.8 µl of a 10 µM solution of each primer for a final volume of 20 µl. Forward (5'-ACG ATG ATA TAG ATC AAA T-3') and reverse (5'-TAC CTA AGC TTC TTG CGT A-3') primers amplify a 111 bp sequence of the merozoite surface protein-1 (MSP-1) gene of *Plasmodium berghei* NK65 and ANKA. DNA was amplified after an initial denaturation of 15 min at 95 °C during 50 cycles with 30 s of denaturation at 95 °C, 45 s of annealing at 52 °C and 30 s of extension at 72 °C. The standard curve was made from a serial dilution of copies of the target sequence (1 to 10^8 copies), which had been cloned previously in *E.coli* plasmids. Each mosquito sample was tested in duplicate in 96 well plates, along with standard dilutions in duplicate, two blanks and one negative control (DNA of uninfected mosquitoes coming from our colony). The final repeatability of our RT-PCR was of 98.2%.

6.3.7 Apyrase activity

The apyrase activity of the salivary glands was determined with a protocol modified from previous studies [168,170]. To obtain a concentration of 0.05 salivary glands per 0.1 ml [168], 1 µl of the salivary homogenate was eluted in 4 µl deionised water, 1 µl of this eluted salivary solution was added to 99 µll 50mM Tris-HCL buffer at pH 9, containing 100 mM NaCl, 5 mM $CaCl_2$, 20 mM β-mercaptoethanol and 20 mM Adenosine S'-Diphosphate (ADP) as substrate in a 96-well ELISA microplate. The mix was incubated for 15 min at 37 C°, when the reaction was stopped with 2 µl of a reducing reagent (0.02% 1-amini-2-naphtol-4-sulfonic acid, 0.12% sodium bisulfite, 0.12% sodium sulfite) and 25 µl 1.25 M H_2SO_4 containing 1.25% ammonium molybdate. The apyrase activity was determined colorimetrically 1 hour after stopping the reaction with an ELISA reader. The standard curve was made as described by

Fiske and Subarrow [171]. Apyrase activity was reported as the quantity of inorganic phosphorous (nmol) produced per minute and salivary glands pairs (1 unit is equal to 1 nmol/min/pair of glands).

6.3.8 Blood quantity

The amount of blood ingested by each mosquito was measured colorimetrically [172]. We ground up the mosquito's abdomen in 1 ml Drapkin solution, added 1 ml chloroform and centrifuged the solution in a horizontal rotor at 660 g for 5 minutes. After this step, 200 µl of the aqueous phase were transferred to a 96-well ELISA microplate. Finally, we measured the absorbance of each sample at 540 nm. The standard curve was made with 0 to 5 µl blood.

6.3.9 Body size

Wing length was used as a proxy of mosquito body size and the distance from the distal end of the alula to the tip of the wing (vein R3) without the fringes was used as a standard measure [148,149].

6.3.10 Statistical analysis

To test whether the sporozoite load differs between the 8 iso-female families, we used a linear model (LM) where the sporozoite load was log-transformed (to follow normal distribution) and set as the response variable, the iso-female family as a factor and the mosquito's wing length as a covariate.

To analyse the effect of infection on apyrase activity, we used a LM. Apyrase activity was log-transformed and set as the response variable, the infection status of mosquitoes (infected or not) and the iso-female family identity were fixed factors, and the mosquito's wing size was a covariate. In order to detect the effect of the intensity of infection, a second analysis was run where infection status was replaced by the log-transformed sporozoite load.

We analysed the size of the blood-meal with a LM, with the infection status, the iso-female family identity and the day of test as fixed factors, and with the time available for mosquitoes to blood-feed, the log-transformed apyrase activity and wing length as covariates. Again, in order to detect an effect of infection intensity, infection status was replaced by the log-transformed sporozoite load in a second analysis. In the latter analysis, the apyrase activity was removed from the model because of collinearity with the sporozoite load.

The proportion of mosquitoes that restarted to probe after a first blood meal was analysed with a generalized linear model (GLM) with a binomial distribution. We only included the mosquitoes that had taken blood 2 hours before. The analysis included the infection status and the iso-female family

identity as fixed factors, the time available for mosquitoes to blood feed, the log-transformed blood-meal size and wing length as covariates. A second analysis was run with the log-transformed blood-meal size replaced by the log-transformed apyrase activity in order to detect any potential effect of apyrase activity on the motivation of mosquitoes to restart biting. Finally, infection status was replaced by the log-transformed sporozoite load in a third analysis.

Finally, the time mosquitoes took to restart probing after their first blood meal was analysed with a Cox proportional hazards regression model (*survival* library in R [128]) for survival analysis. In this analysis, mosquitoes that did not restart probing were censored. The time it took to probe was set as the response variable with the same explanatory parameters used for the analysis of the proportion of mosquitoes resuming probing. A second and a third analysis was also run to detect the effect of apyrase activity and the sporozoite load respectively by following what was done in the previous analysis.

For all analyses, the best model was found by model comparisons. The significance of the effects was assessed with the anova function of the *car* library in R [97], with a type 2 model (if there was no significant interaction) or a type 3 model (if there at least one significant interaction). All analyses were done with the software R (version 3.5.0) [98] and the Rstudio© interface (version 1.1.453) [99].

6.4 Results

Of the 208 mosquitoes that had fed on gametocytic mice, 26 had no sporozoites and were removed from the analysis. We analyzed the behavior of 341 uninfected and 182 sporozoite-positive mosquitoes.

6.4.1 Sporozoite load

On average, infectious mosquitoes harbored 1303 (+/- 485.3 standard Error (SE)) sporozoites. The sporozoite load did not differ among the 8 iso-female families ($F=0.15$, $df=7$, $p=0.99$) and was not correlated with the mosquito's wing length ($F=0.06$, $df=1$, $p=0.81$).

6.4.2 Apyrase activity

Apyrase activity ranged from 1.97 to 20.37 nmol/min/pair of glands and differed marginally among the iso-female families ($F=1.81$, $df=7$, $p=0.08$). In uninfected mosquitoes, the mean apyrase activity was 6.8 (+/- 0.19, SE) nmol/min/pair of glands, while in the infectious ones the apyrase activity was 5.4 (+/- 0.23, SE) nmol/min/pair of glands ($F=34.45$, $df=1$, $p<0.001$). The difference between uninfected and

infectious mosquitos was similar in the 8 iso-female families (F=0.54, df=7, p=0.8). Apyrase activity increased with wing length (F=8.81, df=1, p=0.003) (Fig. 1); there was no effect of the interactions between infection status and wing length (F=1.33, df=1, p=0.25) or iso-female family and wing length (F=1.34, df=7, p=0.23). Finally, for the infectious mosquitoes, there was a negative correlation between their apyrase activity and their sporozoite load (F=26.3, df=1, p<0.001) (Fig. 2).

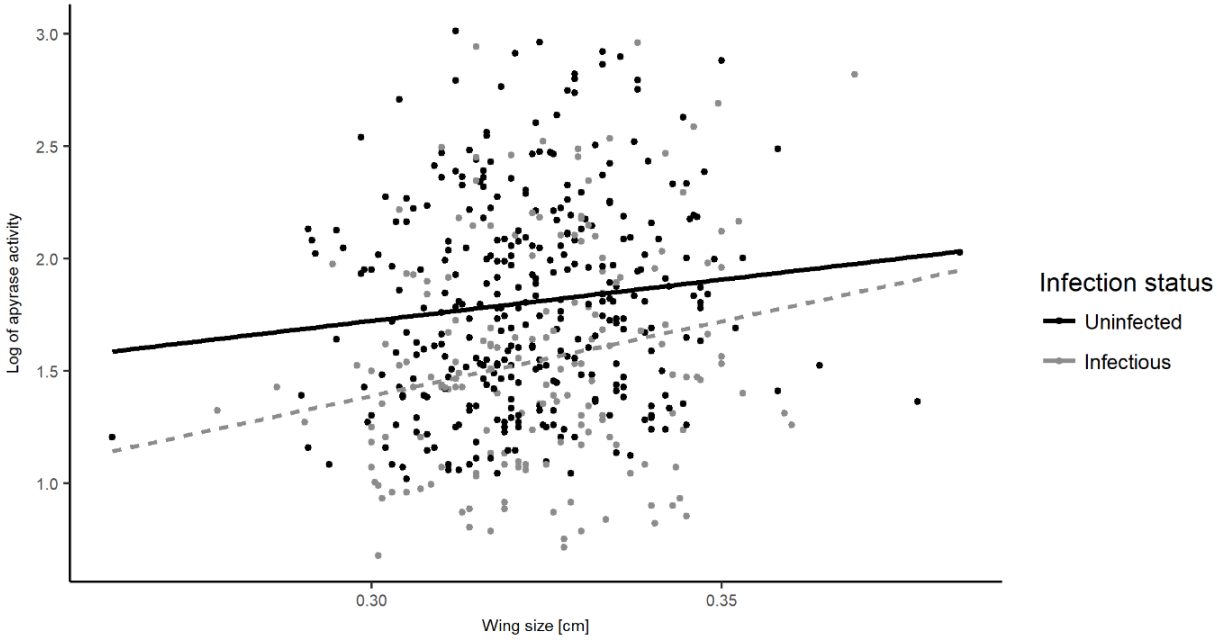

Figure 1: Correlation between the logarithm of apyrase activity and wing length of uninfected (black line and symbols) and infectious (grey line and symbols) mosquitoes. The lines represent the linear regression for each infection status.

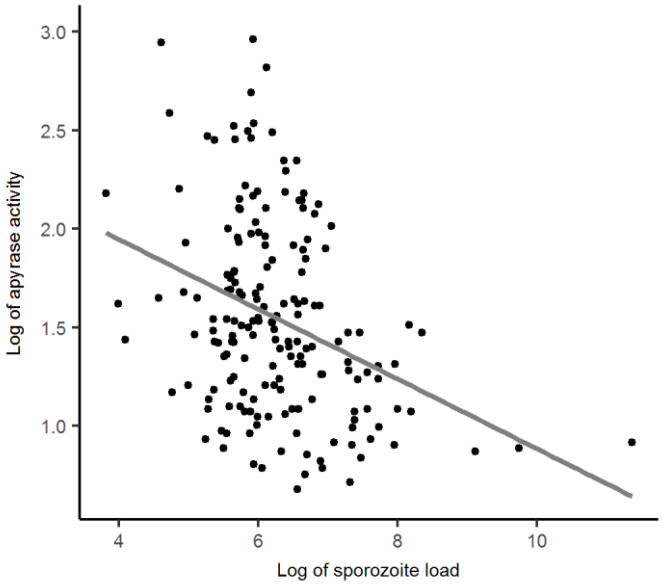

Figure 2: Correlation between log-transformed apyrase activity and log-transformed sporozoite load. The dashed line represents the linear regression.

6.4.3 Blood quantity

The mosquitoes took up between 0.2 and 4.88 µL blood. The blood-meal size increased with the time mosquitoes had to blood feed (F=149.8, df=1, p<0.001), but was not correlated with wing length (F=1.74, df=1, p=0.19). It was similar for the 8 iso-female families (F=1.16, df=7, p=0.33). There was no difference in blood-meal size taken up by infectious or by uninfected mosquitoes (F=1.68, df=1, p=0.2): infectious mosquitoes took up an average of 1.31 (+/- 0.13, SE) µl, and uninfected ones took up 1.44 (+/- 0.11, SE) µl. There was also no effect of the interaction between the iso-female families and the infections status of mosquitoes (F=1.1, df=7, p=0.36). Blood-meal size was, however, positively correlated with the activity of apyrase (F=10.57, df=1, p=0.001) (Fig. 3). There was no difference in the quantity of blood mosquitoes ingested during the 3 days of experiment (χ^2=2, df=1, p=0.16). Finally, in a second analysis, we did not find any effect of the sporozoite load on the quantity of blood mosquitoes took up (F=2.34, df=1, p=0.13).

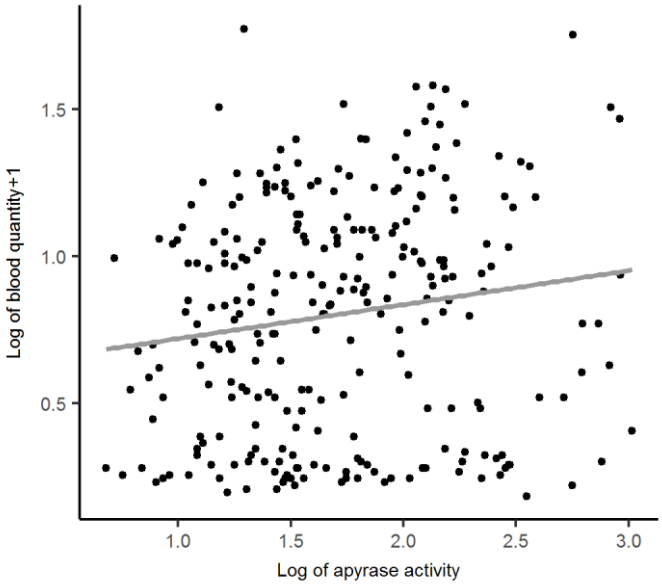

Figure 3: Correlation between the log transformed blood quantity taken up by mosquitoes and their log transformed salivary apyrase activity. The line represents the linear regression.

6.4.4 Motivation to bite after a first blood meal

76.8 % of the mosquitoes tried to bite a second time about 2h after their first blood-meal. The proportion differed among iso-female families (χ^2=15.15, df=7, p=0.03). It was higher if the mosquitoes were infectious (87.5%; 95% confidence interval 79.4-92.7%) than if they were not infected (71.4%; 64.5-77.5%) (χ^2=5.71, df=1, p=0.02), and this difference was similar in the iso-female families (interaction: χ^2=5.45, df=7, p=0.6). It was lower, if mosquitoes had taken up more blood in their first

meal (χ^2=24.78, df=1, p<0.001) (Fig. 4a), but it was not related to wing length (χ^2=2.66, df=1, p=0.1) or the day of the test (χ^2=0.02, df=1, p=0.9). The apyrase activity of mosquito's salivary glands did not affect their motivation to bite (χ^2=0.68, df=1, p=0.4) and finally, the probability of biting a second time was not affected by the sporozoite load (χ^2=0.001, df=1, p=0.99).

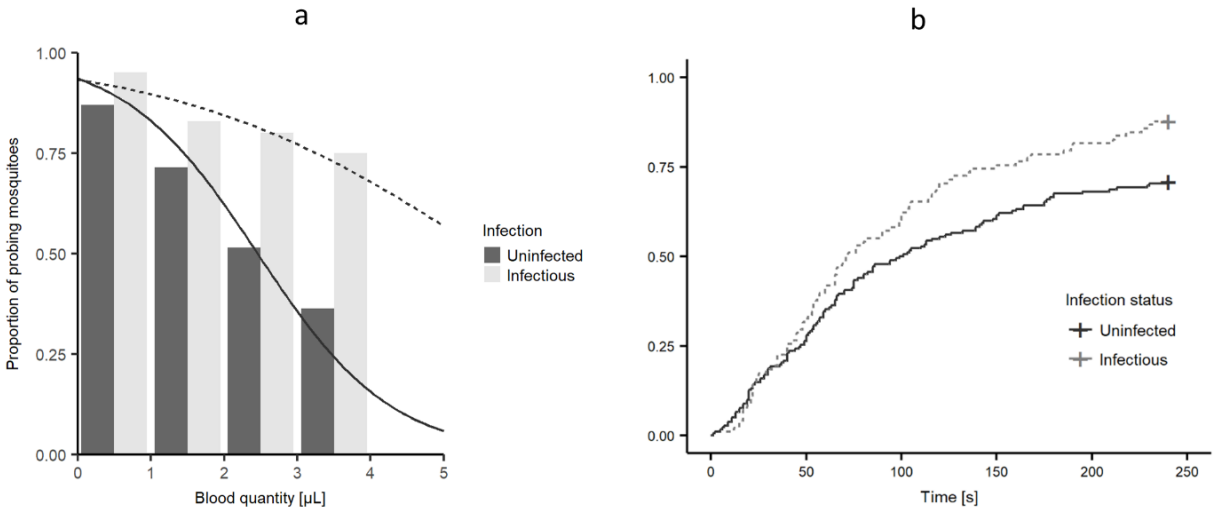

Figure 4: ***a*** *Proportion of probing mosquitoes as a function of blood-meal size. The curves represent the results of the logistic regression (continuous black line for uninfected mosquitoes and dashed grey line for mosquitoes harbouring sporozoites).* ***b*** *Cumulated proportion of mosquitoes that started to probe during their second blood-meal within the time given on the x-axis. The curves show the results of the analysis of the time mosquitoes took to restart probing with the continuous black line for uninfected mosquitoes and the dashed grey one for infectious ones.*

The analysis of the time mosquitoes took to probe during the second trial gave similar results. The time mosquitoes took to resume probing differed among the 8 iso-female families (χ^2=15.39, df=7, p=0.03). On average, infectious mosquitoes took 100.67 (+/- 7.7, SE) seconds to probe and uninfected mosquitoes took 124.42 (+/- 6.6, SE) seconds (χ^2=4.28, df=1, p=0.04) (Fig. 4b); this difference was similar in the iso-female families (interaction: χ^2=4.68, df=7, p=0.7). The less blood mosquitoes had obtained during their first blood-meal, the less time they took to restart to probe (χ^2=20.94, df=1, p<0.001). There was a marginal effect of wing length (χ^2=3.13, df=1, p=0.08), with larger mosquitoes taking more time to resume probing. There was no effect of the day of test (χ^2=0.1, df=1, p=0.76)), the apyrase activity (χ^2=0.72, df=1, p=0.4) or sporozoite load (χ^2=0.17, df=1, p=0.68) on the time it took mosquitoes to probe.

6.5 Discussion

We confirm that malaria parasites influence the biting behaviour of their mosquito vectors [57–59,161]. In particular, using the rodent malaria *P. berghei* and the mosquito *A. gambiae*, we corroborate previous results showing that mosquitoes infected with sporozoites are more motivated to bite than uninfected ones, even if they have taken a blood meal a few hours earlier [54]. We confirm that sporozoite infection decreases the apyrase activity in the mosquito's salivary glands [50] but we also showed that the apyrase activity depends on the sporozoite load, and that the amount of blood a mosquito can take is positively correlated to the apyrase activity. However, we found that the intensity of malaria infection affected neither blood-meal size nor the behavioural traits we measured.

As found for *Ae. aegypti* infected with *P. gallinaceum* sporozoites [54], the sporozoites of *P. berghei* increased the chance of being transmitted by increasing the probability that their mosquito vectors restart biting even if they had already taken a blood meal a few hours earlier. They also increased mosquito's motivation to bite by reducing the time mosquitoes needed to restart that probing [54]. However, neither traits were affected by the intensity of the infection. That sporozoite intensity does not affect the mosquito's host-seeking behaviour corroborates a laboratory experiment of *A. gambiae* infected with *P. falciparum* showing that infection, but not intensity of infection affects probing time and rate [52]. It contrasts, however, a result from the field, which suggested that more intense infection leads to more frequent biting [57]. This difference may be due to the complexity of blood-feeding behaviour. While spozoroite load may not affect the probing behaviour of mosquitoes, it may affect their biting rate via other mechanisms. Indeed, by reducing the apyrase activity of their mosquito hosts, which affected their blood-meal but not their motivation to bite, sporozoites decrease the mosquito's ability to feeding. Since low activity of apyrase reduced the chance for a mosquito to take a complete blood meal, infectious mosquitoes would have to try to blood-feed many times to obtain as much blood as they want. Since apyrase activity was linked to sporozoite load, the tendency to feed multiple times is expected to be linked to sporozoite load [51], as observed in the field [57].

It may therefore seem surprising that we found no correlation between the sporozoite load and the blood quantity in our study, in particular since an earlier study reporting that *Ae. aegypti* mosquitoes infected with *P. gallinaceum* sporozoites take less blood than uninfected ones during a fixed amount of time [54]. A possible bias may come from the short amount of time we gave to mosquitoes to take blood. Indeed, we may have needed more time to observe the effect of the reduced apyrase activity on the feeding capacities of the infectious mosquitoes, thus to see them interrupt their blood meal and restarting probing.

That parasites manipulates the behaviour of their vectors by altering their efficiency to take blood seems to be a general feature in vector-borne diseases [173]. Indeed, it has been observed for sandflies infected with different *Leishmania* species [174], tse-tse flies infected with *Trypanosoma* spp. [175], *Rhodnius prolixus* bugs infected with *Trypanosoma rangeli* [176] and the rat flea *Xenopsylla cheopsis* infected with *Yersinia pestis* [177]. What is specific is the mechanism underlying this behavioural change. While *Leishmania* and *Y.* pestis physically block the uptake of blood and *Trypanosoma spp.* alter the saliva of tse-tse flies, *Plasmodium* and *Trypanosoma rangeli* change the activity of apyrase in the saliva of mosquitoes and kissing bugs respectively.

As a second aim, we wanted to evaluate whether the potential of mosquitoes to be manipulated by malaria parasite has a genetic basis. Although the apyrase activity, the proportion of mosquitoes restarting probing few hours after a first blood meal and the time they needed to restart that probing were marginally different among the iso-female families, the effects of sporozoites on these traits were not. Thus, at least for the traits we measured and for our rodent malaria model, the sensitivity of mosquitoes to be manipulated by malaria parasite has no genetic basis. One explanation for this lack of a genetic basis could simply be that the mosquitoes of our study have not coevolved with *P. berghei*. A more interesting one would be that the parasites have evolved a way to circumvent any genetic resistance of the mosquitoes against being manipulated.

Overall, our results confirmed that sporozoite infection modify the host-seeking behaviour of mosquitoes. The apyrase activity was found to play a role in the feeding capacities of the mosquitoes, by determining the quantity of blood they were able to take. More interestingly, the apyrase activity was found to be not only modified by sporozoite infection but was also negatively correlated with the amount of sporozoites. These results thus confirm that similarly to other vector-borne diseases, malaria parasites seem to increase their chance of transmission by modifying the feeding abilities of the mosquitoes. In conclusion, our results will give new insights in understanding how malaria parasites may affect the behaviour of their mosquito vectors in a way to increase their own transmission and may be helpful in the context of malaria control.

Chapter 7: General discussion

7.1 Summary of results

7.1.1 Chapter 2

In this chapter, we first evaluated whether sub-lethal exposure to permethrin may affect mosquito's host-seeking behaviour. In a second step, we tested if mosquitoes infected with malaria were affected similarly to uninfected mosquitoes to these sub-lethal exposures. Finally, because it determines the doses of insecticide a mosquito might receive, we compare the contact irritancy between infected and uninfected mosquitoes. We found that short exposure to permethrin inhibited the feeding motivation of mosquitoes for almost 48 hours. Although malaria infection did not affect the contact irritancy, it shortened the inhibition of feeding behaviour induced by permethrin independently of the infection stages (the developmental one, i.e. oocysts, or the infectious one, i.e. sporozoites). On the one hand, the inhibition of mosquito's feeding behaviour may increase ITNs efficacy by first decreasing the chance that uninfected mosquitoes encountered the malaria parasites and second by decreasing the chance that infectious mosquitoes transmit their sporozoites (although the inhibition was weaker for them). On the other, that oocyst infection shortened the inhibition may increase the chance that mosquitoes survive long enough to develop sporozoites and thus become infectious. Overall, the results illustrate how complex the interactions between insecticides and malaria infections are and may help to fully understand the real impact of ITNs on malaria transmission.

7.1.2 Chapter 3

Classical techniques to evaluate the efficacy of insecticide treated bed nets generally test whether mosquitoes are able to pass through the net and to take blood afterwards [28–31]. In the third chapter, we tested another possibility; that mosquitoes are able to bite directly through an ITN. We also recorded the behavioural and physiological aspects of such a blood meal. This experiment reveals that at least at short range a commercially available permethrin-treated bed net (Olyset plus®) prevent that mosquitoes bite directly through the net by only 12%. It thus suggests that when touching the net, the users loose almost all the personal protection offered by the net. However, taking a blood meal through an ITNs have fitness costs for mosquitoes. Indeed, by staying much less longer on the treated net, mosquitoes took less blood and it resulted in a reduced number of eggs produced. In addition to

fecundity costs, biting through the treated net decreased the short-term survival of mosquitoes (24 hours after the blood meal) but did not reduced their longevity. Overall, our results suggest that when having the possibility, most of the mosquitoes may be able to bite through an ITN, this increasing the probability of the user to become infected. However, the reduction in mosquito's survival after such a blood meal may contribute to maintain a certain level of the community-wide protection, for the mosquitoes may die before they become infectious.

7.1.3 Chapter 4

In the fourth chapter, we investigated whether the genes responsible of pyrethroids resistance are the same that make mosquitoes less spatially repelled or irritated. In other words, with an evolutionary experiment, we evaluated if resistance, spatial repellency and contact irritancy are genetically correlated in mosquitoes. During this experiment, we were able to select mosquitoes to be: 1) partially resistant, 2) strongly irritated and 3) weakly irritated. We were, however, not able to select mosquitoes to be strongly or weakly spatially repelled and thus not able to evaluate if that trait was genetically correlated with the two others (i.e. resistance and irritancy). Our results showed there was no genetic correlation between resistance and contact irritancy. Indeed, first, we confirmed the resistance to decrease irritancy and second, we found not irritated mosquitoes to be as susceptible as the control ones. We thus discover that other genes than the ones that cause resistance decreases the insecticide's irritancy. Both the genes of resistance and the ones that make mosquitoes not irritated may decrease the personal protection offered by ITNs, for they may increase the chance that mosquitoes pass or bite through the net. However, it also suggests that resistant and not irritated mosquitoes may pass enough time on the net to receive a dose of insecticide that may kill them, thus increasing community-wide protection [29,44,45].

7.1.4 Chapter 5

In the fifth chapter, we evaluated if the repellency of permethrin-treated net is different between uninfected mosquitoes and mosquitoes infected with sporozoites (the malaria stage that is infectious for human). Using two different measure of repellency and two different malaria systems, we found the sporozoite infection to decrease the repellency of permethrin-treated nets. First, in the laboratory at the University of Neuchâtel, we found *A. gambiae* mosquitoes infected with sporozoites of the rodent malaria *P. berghei* to choose indifferently between a cage containing a permethrin-treated net or a cage containing an untreated net while uninfected mosquitoes chose preferentially the cage without the insecticide. Second, in the laboratory at the Bagamoyo Research and Training unit (Tanzania), we found *A. arabiensis* mosquitoes infected with sporozoites of the human malaria parasite

P. falciparum to be more likely to pass through a commercially available permethrin treated net (Olyset) compared to uninfected mosquitoes. This reduced repellency of ITNs for infectious mosquitoes suggest that sporozoite infection decreases the personal protection offered by the treated net. In contrast, by trying more often to pass through the ITN, infectious mosquitoes may receive higher dose of insecticide and thus may suffer from higher mortality, this increasing the community protection. However, even if it was marginally increased during our experiment, the difference in mortality between infectious and uninfected mosquitoes was far from significant. This result may be explained by the fact that malaria infected mosquitoes seems to recover faster from sub-lethal exposure to permethrin (see chapter 2). Overall, with this experiment we showed that malaria infection decreases the personal protection of ITNs' users at the stage where it is the most needed.

7.1.5 Chapter 6

Sporozoite infection alter the mosquito's host-seeking behaviour in several ways, making them globally more motivated to blood feed [58,59]. In the sixth chapter of this thesis, we first investigated the role of sporozoite load and apyrase activity on mosquito's feeding capacities and behaviour. As a second objective, we investigated whether the potential of being manipulated as a genetic basis in mosquitoes. The results first confirmed that sporozoite infection increases the motivation of mosquitoes to restart probing even if they took blood few hours before [54]. They also confirm that the malaria parasites, like other vector-borne parasites [173], modify the feeding capacities of their mosquito vectors by altering their saliva. In particular sporozoites reduce the activity of the apyrase, an enzyme that help mosquitoes to locate blood. In addition, the activity of apyrase greatly depended on the degree of sporozoite infection (sporozoite load) with the higher the number of sporozoites was the lower was the apyrase activity. By impeding mosquitoes to efficiently take blood, sporozoites may increase the probing time [50] and bite rate [51] of mosquitoes and reduce the chance they took a complete blood meal, leading to an increased chance they bite multiple hosts [51]. However, in this study the sporozoite load did not modify neither the quantity of blood the mosquitoes were able to take or their motivation to bite confirming previous results that found no correlation between sporozoite density and probing time and biting rate [52]. Finally, the results of this chapter revealed no genetic basis for the potential of being manipulated by malaria parasites in mosquitoes (at least for the behavioural traits we measured and the malaria system we used). Overall, the results emphasized that malaria parasites modify the mosquito's behaviour by decreasing their feeding capacities.

7.2 Future perspectives

7.2.1 Chapter 2-5

In the second chapter we found sub-lethal dose of insecticides to inhibit the feeding motivation of mosquitoes. This inhibition was however reduced for malaria infected mosquitoes at both developmental and infectious stages, suggesting that malaria infected mosquitoes may recover faster from sub-lethal exposure to permethrin. Because this may weaken the efficacy of ITNs, further studies may evaluate how malaria infected mosquitoes recover from permethrin exposure. As a first step, we suggest evaluating the role of oxidative stress. Indeed, both malaria infection [101–104] and pyrethroids exposure [105,106] are strongly linked with the production of reactive oxygen species (ROS) in mosquitoes.

The results of the third chapter suggest that when evaluating the efficacy of ITNs to protect the users, we may benefit from considering that when having the possibility, most of the mosquitoes (88%) are trying to directly bite through a permethrin-treated net. Indeed, it suggest that when touching the net, the users loose almost all the personal protection offered by the ITNs. A further step may be to evaluate how malaria infection may affect the ability of mosquitoes to take blood through an ITNs. Indeed, because it reduces insecticide's repellency (chapter 5) and because it may make mosquitoes recover faster from insecticide's exposure (chapter 2), malaria infection may increase the chance that mosquitoes bite through an ITN and may increase the probability they survive after such a blood meal.

In the third chapter, we also found that the reduction in survival 24 hours after the blood meal through the Olyset plus net was not as strong as we expected from the time-response curve. We expected the blood meal to help mosquitoes to deal with the insecticides, in particular the fact that permethrin exposure and blood feeding occurs simultaneously. Thus, a further step of this experiment may be to investigate the role of oxidative stress and temperature on the ability of mosquitoes to survive a blood meal through an ITN. Indeed, first, both blood meal [101] and pyrethroid exposures increase the production of ROS [105,106]. Thus, when not occurring simultaneously, they may reduce the survival of blood fed mosquitoes compared to unfed ones, for the production of ROS they induce may be cumulated. Second, because the toxicity of permethrin decreases while temperature increases [135–137], we expect the increased temperature of mosquito's body during the blood meal [134] to explain why when being simultaneously exposed to permethrin and taking blood mosquitoes may survive better.

In the fourth chapter, we showed that although pyrethroid resistance decreases the contact irritancy of deltamethrin, there was no genetic correlation between the two traits. More surprisingly, we found the mosquitoes selected to be strongly irritated to be more resistant than the one selected to be not irritated. As resistance may reduce the efficacy of ITNs, a first next step could be to identify which mutation of resistance make mosquitoes less irritated. In addition, it may be interesting to identify the gene responsible of weak irritancy in our experiment. Indeed, by selecting not irritated mosquitoes, irritancy may increase the number of mosquitoes that are able to pass or bite through the net, thus the one that may be able to transmit their parasite despite the presence of ITNs.

Because malaria infection also decreases the personal protection insecticide treated bed nets offer, it may be essential to test if it may affect how resistance decreases the insecticides irritancy. Indeed, in the fifth chapter, our permethrin-treated net loses its ability to prevent mosquitoes from passing through when these were both partially resistant and infected with sporozoites of *P. falciparum*. This result therefore suggests that when combined, resistance and malaria infection may supress the personal protection offered by the ITNs at the stage where it is the most needed.

Overall, further theoretical and empirical experiments that may evaluate the epidemiological impact of ITNs on malaria, must consider that:

1) irritated mosquitoes may not be able to restart their host research immediately after the first tentative, for the sub-lethal exposure that follow irritancy inhibits their blood feeding motivation;
2) mosquitoes may be able to directly bite through the net and to survive afterwards;
3) pyrethroid resistance decreases the personal protection they offer by decreasing both spatial repellency and contact irritancy;
4) malaria infection reduces both the inhibition of host-seeking behaviour induced by sub-lethal exposure and the repellency of insecticides;
5) malaria infection may help mosquitoes to deal with insecticides.

7.2.2 Chapter 6

The results of the chapter six revealed that like for other vector-borne parasites [173], malaria infection impede the feeding capacities of their mosquito vectors by modifying their saliva. We also showed the sporozoite load to be negatively correlated with the apyrase activity in the salivary glands of mosquitoes. Further studies must evaluate whether this is also true for a realistic human-malaria model. Indeed, the behavioural alterations induced by malaria parasites may diverge in function of the malaria system [161]. For the same reasons, when evaluating if malaria parasites manipulates their

mosquito vectors, it may be necessary to standardize the methodology in order to produce more robust conclusions. For example, here using the same process but a different malaria system than in a previous study [54], we confirmed that sporozoite infection increases the likelihood that mosquitoes restart blood feeding even if they took a first blood meal few hours before.

Further studies on behavioural manipulation of mosquito by malaria parasites may also evaluate the ecological and evolutionary aspects of such a manipulation. Indeed, as behavioural manipulation certainly help to maintain intense transmission of malaria, we may benefit from knowing whether parasite's ability to manipulate mosquitoes is influenced by its genotype (genetic variation is the prerequisite for the evolution of manipulation), by host genotype (host immune capacity affect parasite infection) or by any environmental factors (environmental variation affects the evolutionary pressure) [178].

7.3 Conclusions

Overall this thesis revealed how complex the evaluation of the efficacy of insecticide treated bed nets may become when considering that the behaviour of mosquitoes may be altered by sub-lethal exposures, malaria parasites, and resistance to insecticides. Globally, the topics covered in this thesis are relevant for different areas of evolutionary ecology, particularly for the epidemiology of mosquito-borne diseases, and they may have implications for future research in that precise field. The main results of this thesis revealed for the first time that malaria parasites may affect their own control by altering the behaviour and physiology of their vectors with regards to the method of control. Finally, although this thesis contributed to a better understanding of the real epidemiological impact of insecticide-treated bed nets on malaria, many questions remains.

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