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Effects of sublethal doses of an insecticide on the vectorial capacity of the malaria vector *Anopheles gambiae*

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Abstract

The presence of insecticide residues in water can strongly affect the development and life-history of mosquitoes. As the toxicity usually decreases along with the concentration, it is often considered that sub-lethal doses of insecticides induce negligible effects. However, there is increasing evidence that when applied at low doses, toxic substances may induce substantial and unpredictable effects on the organisms. Since these effects may affect the ability of mosquitoes to transmit pathogens, the study of sublethal doses in the context of vector ecology is of both an eco-toxicological and an epidemiological interest.

In this thesis, the malaria mosquito vector and a pyrethroid insecticide were used to experimentally assess the consequences of low doses of insecticides on several aspects of mosquito's vectorial capacity. In the different experiments, mosquitoes were mainly – but not only – exposed at larval stage to test for possible carry-over effects on the adults. In the first four chapters, the effect of the insecticide on mosquito's development and adult life-history traits (Chap. 2), competence for the malaria parasite (Chap. 3), immunity (Chap. 4), and oxidative state (Chap. 5) were examined. In the sixth chapter, the possibility and consequences for mosquitoes to bite through an insecticide-treated bed-net (ITN) were explored. Finally, an evolutionary approach was used to test the possibility for both the insecticide at sublethal dose and the contact with an ITN to contribute to the evolution insecticide resistance (Chap. 7). As the response of mosquitoes to the insecticide may vary following environmental conditions, the effect of larval competition was additionally assessed as a proxy for limited resources. The results and their implications for both malaria transmission and the evolution of insecticide resistance are discussed throughout the thesis.

Overall, this work provides a broader understanding of the physiological, epidemiological, and evolutionary consequences of the exposure of mosquito larvae to insecticide residues, and stresses the need for a better consideration of sub-lethal doses in the field of vector-borne diseases.

Keywords

Pyrethroid insecticides, *Anopheles gambiae*, sublethal effects, vector control, insecticide resistance, malaria, vectorial capacity, oxidative stress

Résumé

La présence de résidus d'insecticide dans l'eau peut avoir un fort impact sur le développement et le cycle de vie des moustiques. Puisque la toxicité décroît généralement avec la concentration, il est souvent considéré que les doses sous-létales d'insecticide n'induisent que des effets négligeables. Cependant, de plus en plus d'éléments tendent à montrer que lorsqu'elles sont appliquées à faible doses, certaines substances toxiques peuvent provoquer des effets substantiels et imprévisibles sur les organismes. Etant donné que ces effets peuvent affecter la capacité des moustiques à transmettre des agents pathogènes, l'étude des doses sous-létales dans le contexte de l'écologie des vecteurs présente non seulement un intérêt éco-toxicologique, mais aussi épidémiologique.

Dans cette thèse, le moustique vecteur du paludisme et un insecticide pyréthrinoïde ont été utilisés pour évaluer expérimentalement les conséquences des doses faibles d'insecticide sur plusieurs aspects de la capacité vectorielle du moustique. Dans les différentes expériences, les moustiques ont été principalement – mais pas seulement – exposés au stade larvaire pour tester d'éventuels effets de report sur les adultes. Dans les 4 premiers chapitres, l'effet de l'insecticide sur le développement et le cycle biologique du moustique (Chap. 2), sa compétence pour le parasite responsable du paludisme (Chap. 3), ses défenses immunitaires (Chap. 4), et son stress oxydant (Chap. 5) ont été étudiés. Dans le sixième chapitre, la possibilité et les conséquences pour les moustiques de piquer à travers une moustiquaire imprégnée d'insecticide ont été explorées. Finalement, une approche évolutive a été utilisée pour tester la possibilité qu'un insecticide à dose sous-létale et/ou un contact avec une moustiquaire imprégnée contribue à l'évolution de la résistance à l'insecticide chez le moustique (Chap. 7). Comme la réponse du moustique face à l'insecticide peut varier en fonction des conditions environnementales, l'effet de la compétition entre les larves pour des ressources limitées a également été évalué. Les résultats et leurs implications tant pour la transmission de la malaria que l'évolution de la résistance à l'insecticide sont discutés tout au long de la thèse.

Dans l'ensemble, ce travail permet de mieux comprendre les conséquences physiologiques, épidémiologiques et évolutives de l'exposition des larves de moustiques à des résidus d'insecticide, et soulignent la nécessité de mieux tenir compte des doses sous-létales dans le domaine des maladies transmises par des vecteurs.

Mots-clés

Pyréthrinoïdes, *Anopheles gambiae*, effets sous-létaux, lutte antivectorielle, résistance aux insecticides, paludisme, capacité vectorielle, stress oxydant

Contents

List of abbreviations

ABBREVIATION	DESCRIPTION
DDT	dichlorodiphenyltrichloroethane
IRS	indoor residual spraying
ITNs	Insecticide-treated bed-nets (used equally to speak about long-lasting insecticide-treated bed-nets (LLINs))
WHO	World Health Organization
ACh	acetylcholine
AChE	acetylcholinesterase
OC	DDT-type organochlorines
OP	organophosphates
CA	carbamates
PY	pyrethroids
kdr	knock-down resistance
CNS	central nervous system
ppm	parts per million
ROS	reactive oxygen species
OS	oxidative stress
ANOVA	analysis of variance
GLM(M)	generalized linear (mixed-effect) model
ZINB	zero-inflated negative binomial model
ID	identity/identifier
v/v	Volume per volume
w/w	weight per weight

Chapter 1

General Introduction

1.1 Background

1.1.1 Malaria and vector control

Malaria is a harmful and potentially deadly mosquito-borne disease caused by protozoan parasites of the genus *Plasmodium*. In 2017, malaria parasites infected 203 to 262 million people worldwide and caused the death of 435'000 of them, mostly children under 5 years old (61%) (1). Malaria alone is responsible for more than 60% of the mortality caused by vector-borne diseases in the world (2). Its impact on human populations is strong enough to induce a measurable selection pressure for resistance allele in endemic areas (3). Malaria burden is especially important in Africa, where 92% of the cases are reported. Other regions impacted are South-East Asia (5%), Eastern Mediterranean (2%), Western Pacific and Americas (1%) (1).

Human malaria is caused by 4 different *Plasmodium* species: *P. falciparum* (97% of malaria cases), *P. vivax, P. malariae, and P. ovale* (4). Some rare cases of malaria caused by a fifth species usually found in macaques, *P. knowlesi*, have also been reported (1, 5). All of the parasite species are transmitted to humans exclusively by *Anopheles* spp. mosquitoes. The genus *Anopheles* contains more than 450 species (6), out of which 70 are competent for malaria (4). Mosquito females can acquire the parasite when they take a blood meal on an infected host during which they ingest *Plasmodium* gametocytes. It takes about 8-16 days for the parasite to complete its development and be ready to be transmitted to a new host via mosquito saliva (4) (described in **Fig. 1**).

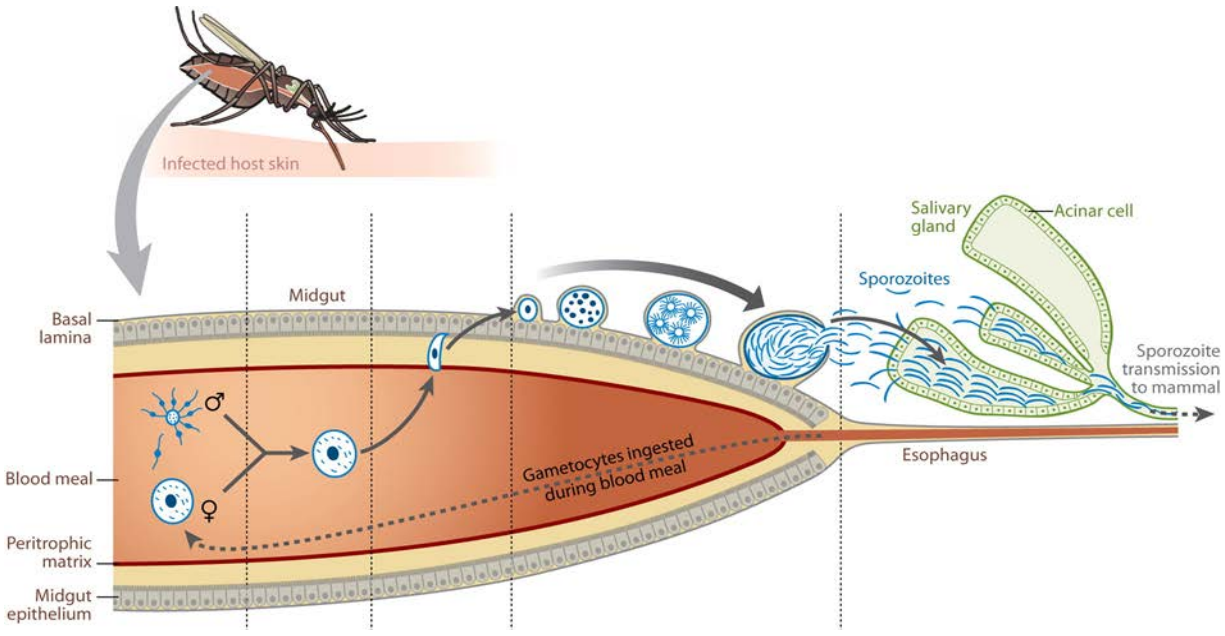

Figure 1. The malaria cycle inside its mosquito host. After being ingested, male and female gametocytes merge into a zygote. It then transforms into a motile ookinete that crosses the midgut epithelium. The ookinete attaches itself to the midgut wall and pursues its transformation into an oocyst. A single oocyst produces thousands of haploid sporozoites. After rupture of the oocysts, the sporozoites are released in the hemocoel and transported by the hemolymph to the salivary glands. Adapted from *(7)*.

The complex development of the malaria parasite inside its mosquito host implies that its epidemiology is very sensitive to vector control strategies. Today, vector control tools is, with chemoprevention drugs, one of the two pillars of the World Health Organization (WHO) malaria prevention program (1). Historically, vector control programs started at the beginning of the 20th century with large scale spreading of the highly toxic "Paris Green" (a mixture of arsenic, copper and acetic acid (8)), replaced in 1946 by the organochlorine insecticide dichlorodiphenyltrichloroethane, better known as DDT (9). Epidemiologically, this strategy was an indisputable success: malaria death rate went from 6 million per year in the late 1930s to 2.5 million in 1965 (10). However, the large-scale spreading of DDT was strongly prejudicial for the environment: it killed many non-target insects, intoxicated soil and aquatic organisms, and led to the bioaccumulation of toxic compounds in animals across the food chain (11, 12). Birds were particularly affected: DDT metabolites were found to alter their calcium metabolism, resulting in thinner and fragile eggshells that caused the death of many embryos (13, 14). In addition, DDT affected living organisms for years after it has first been spread; its half-life is estimated to be 2 to 15 years (15).

The publication by Rachel Carson of the book *Silent spring* in 1962 (16), whose title evokes a spring without birds, raised awareness of the population to the ecological and possible health issues caused by this insecticide. These concerns and the apparition of resistance to DDT in *Anopheles* mosquitoes (17) together led to a more varied, reasoned and targeted application of insecticides for vector control. Large scale spreading was progressively replaced by indoor residual spraying (IRS) and the use insecticide-treated bed nets (ITNs[1]). IRS kills or repels mosquitoes out of dwellings, while ITNs create a physical and chemical barrier that protects the user. Both techniques significantly contributed to reduce malaria prevalence (18, 19), and still constitute the core interventions recommended by the WHO today (1). The environmental and health concerns about the use of insecticides led to the adoption of the concept of *Integrated Vector Management* by WHO in 2004, which was applied to its global vector control strategy. This concept was defined as "a rational decision-making process for the optimal use of resources for vector control" (20).

Regardless, DDT is still used for IRS (21) for its advantageous cost-effectiveness as well as its lasting insecticidal and repellent properties, but is no more spread on mosquito breeding sites and has been banned from agricultural practices in most countries (22).

1.1.2 Insecticides in agriculture

During the same period, pesticides (including insecticides) were also extensively used in agriculture. And, similarly to vector control strategies, agricultural practices also had to adapt following the global

[1] In the present work, the abbreviation "ITNs" is used as a general term to refer to both *insecticide-treated bed-nets* and *long-lasting insecticidal nets* (LLINs), in which the insecticide is incorporated into the net fabric.

overuse of DDT and other organochlorines in the middle of the last century. This transition was described by Metcalf in 1980, who separated what he called the *Age of Pesticides* in agriculture in three eras (23). First, the *Era of Optimism* (1946-1962) during which DDT was used extensively and was thought to be the ultimate solution against pest insects. Second, the *Era of Doubt* (1962-1976), which was initiated by the book *Silent spring* and the rapid evolution of resistance in pests. And third (since 1976), the *Era of Integrated Pest Management,* which aims to respond to these issues by replacing systematic spreading by interventions based on monitoring and economic thresholds, and by integrating prevention methods to limit the use of pesticides. Thus, DDT was progressively abandoned for agricultural practice to the benefit of other insecticide classes, mainly organophosphates, carbamates, and pyrethroids (17). However, contrary to vector control, the spreading of insecticide remained a crucial tool in agriculture (17).

Nowadays, agriculture is still the principal user of insecticides worldwide with, each year, ca. 350'000 tons of active ingredient, against ca. 12'000 tons for vector control (data for the year 2009, estimated from (24, 25)). At the global scale, it has been calculated that each hectare of crop receives an average of 220 grams of insecticide per year (26), from where the chemical compounds may be dispersed in the environment, mainly through atmospheric deposition, runoff, or soil leaching (27). Hence, despite the efforts made, insecticide residues (mostly organochlorines, organophosphates and pyrethroids) are found in most surface waters and sediments around the world, the highest concentrations being usually found in Africa, South-America, and Asia (28).

1.1.3 Biological impact of insecticides

Most insecticides are neurotoxic compounds that disrupt nerve impulses, usually leading to an overexcitation of neurons. The mode of action of the four main insecticide classes used for vector control, that is DDT-type organochlorines (OC), organophosphates (OP), carbamates (CA), and pyrethroids (PY), is described in **Figure 2**. In addition, OP, CA, and PY are still used in agriculture, and evidences suggest that OC continue to be used illegally in developing countries (29).

By affecting insects' peripheral and central nervous system, these insecticides first induce behavioral intoxication symptoms, such as tremors, convulsions, hyperactivity followed by paralysis, and eventually death (30–32). By killing insects, insecticides select for resistance mechanism, which may evolve in very short time: first evidence of resistance to DDT in *Aedes* mosquitoes were observed only 1 year after the chemical was first used to control mosquito vectors (33). For OC, OP, CA and PY, resistance is mainly conferred by two mechanisms: increased detoxification, called *metabolic resistance*, and mutations at the binding site of the toxic molecules, called *target-site resistance* (described in (34)). For target-site resistance of voltage-gated sodium channels (**Fig. 2**) that confer resistance to pyrethroids or DDT insecticides, it is more commonly referred to *kdr* for "knock-down

resistance". Moreover, in pyrethroids, a third type of resistance conferred by the thickening of the insect's cuticle has been also described (35–37). The exposure pathways and resistance mechanisms for pyrethroids are shown in **Figure 3**.

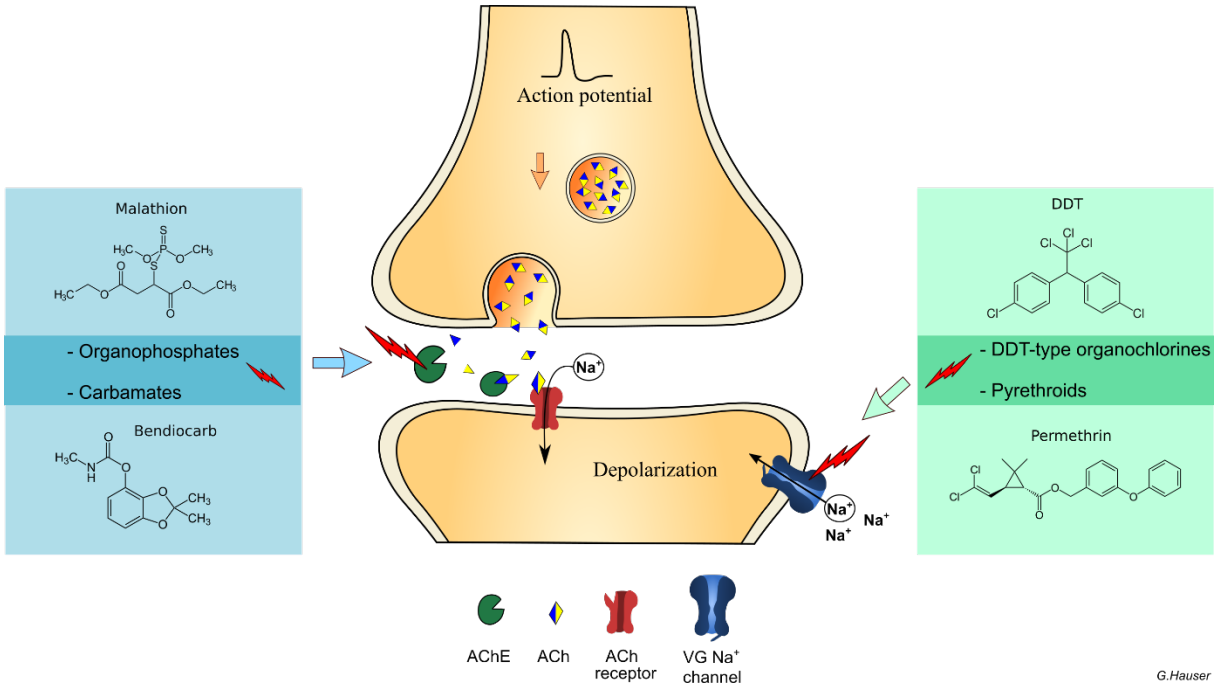

Figure 2. Mode of action of organophosphate, carbamate, DDT-type organochlorine and pyrethroid insecticides. Shown is the transmission of a nerve impulse (action potential) via the synaptic release of acetylcholine (ACh). Ach binds to the acetylcholine receptor (ACh receptor, in dark red) of the post-synaptic cell, which opens and lets sodium ions (Na^+) enter the cell. This release triggers a depolarization. Organophosphates and carbamates (left side) bind to acetylcholinesterase enzymes (AChE). They prevent the breakdown of ACh and induce the overexcitation of neurons. DDT-type organochlorines and pyrethroids (right side) bind to the voltage-gated sodium channels (VG Na^+ channel, in dark blue) and prevent them for closing, resulting in repeated action potential and neurons overexcitation.

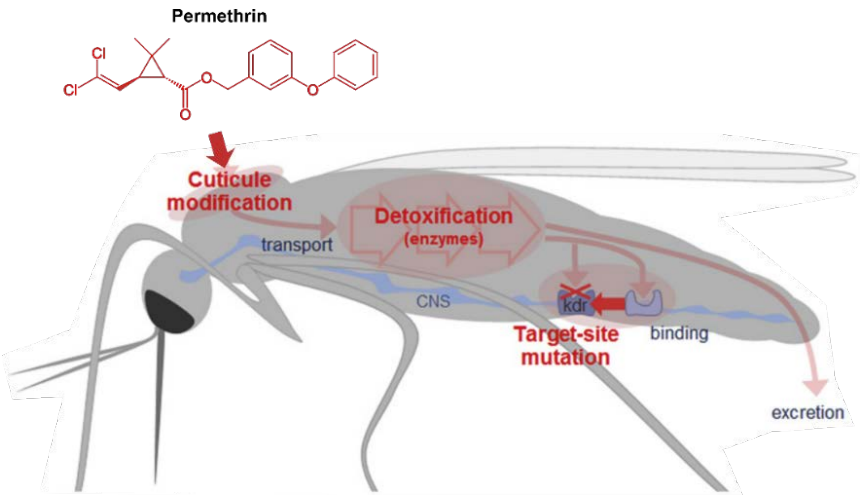

Figure 3. Route of exposure and major resistance mechanisms of pyrethroid insecticides. The insecticides pass through the cuticle to reach the peripheral and central nervous system (CNS). Resistance mechanisms represented are, from left to right: cuticle thickening reducing the penetration of the toxic compound, enhanced detoxification (metabolic resistance), and target-site mutation (kdr) preventing the insecticide to bind to the sodium channel. Adapted from *(35)*.

When insecticides are released in the environment, their concentration may greatly vary and decrease over time through dilution and degradation. Therefore, insecticide residues are also likely to affect organisms in a sublethal way. For example, neurotoxic insecticides may directly alter the behavior of insects by irritating (OC, CA, and PY) or repelling them (mainly OC, to a lesser extent PY and CA) (38–42). Further behavioral impairments include altered mating or reproductive behavior (43, 44), host-seeking and feeding behavior (44–49), and orientation or locomotion (44, 50, 51). Finally, insects exposed to insecticides may also suffer from indirect effects on important life-history traits. For instance, insecticides were found to affect larval development (52–54), adult size (55), longevity (43, 53, 56), fecundity (41, 46, 53, 55), and immunity (reviewed in (57)).

Despite the large number of studies depicting the sublethal effects of insecticides on nearly all aspects of insects' life, the effects of sublethal concentrations on these traits are still largely overlooked. To avoid confusion, *sublethal effects* and *sublethal concentration* must first be defined. I use here the definitions proposed by Desneux et al. in 2007 (58), who defined sublethal effects as "effects (either physiological or behavioral) on individuals that survive exposure to a pesticide", and sublethal dose/concentrations as a dose "inducing no apparent mortality in the experimental population". Thus, while the former has been largely documented with, in most cases, partially lethal doses, the effects induced by the latter – strictly sublethal concentrations – remain largely unexplored.

There are at least two reasons why the consequences of sublethal and partially lethal doses should be tested and interpreted separately. First, a dose that kills a part of the population not only induces sublethal effects, but also selects the most resistant individuals. As resistance is likely to be correlated to some physiological or phenotypic traits (e.g. body size (59)), the selected individuals may come with some characteristics that can be confounded with (or hide) a physiological or behavioral effect induced by the insecticide. A probable example of such confounding effects have been shown in *Aedes aegypti* larvae that were exposed to a partially lethal concentration (LC_{50}) of an insecticide (60). Unexpectedly, it was found that surviving adults were larger and laid more eggs, which is the opposite of what was found with lower doses of the same insecticide (61). While such studies are important and ecologically relevant, they do not allow to explain the relative contribution of both the sublethal and the lethal (i.e. what the insecticide selected for) effects of the insecticide.

Second, evidences suggest that at very low dose, a toxic insecticide may actually have beneficial and not detrimental consequences on an organism (e.g. (62–64)). This biphasic dose-response induced by an environmental perturbation that is beneficial at low intensity and detrimental at high intensity is known as hormesis (65). A hormetic response is typically expected when a perturbation of low intensity elicits a physiological response that overcompensate the initial detrimental effect (66). As such

compensatory mechanisms can be triggered by various environmental perturbations and in different organisms – including plants (67) – it has been argued hormesis to be a highly generalizable phenomenon (68).

Cutler reviewed in 2013 the current knowledge on hormetic effects induced by low doses of insecticides (69), but, unfortunately, none of the study reported was done with disease vectors. However, he reported (on other insects) positive effects of all four classes of insecticides used for vector control, such as higher growth rate (all classes), increased fecundity (all classes), increased longevity (OC and CA), and increased egg viability (PY). Studies focusing on mosquito vectors are much more scarce (e.g. (55)). Recently, the effect of increasing doses of spinosad, a natural insecticide derived from bacteria, was tested on the mosquito vector *Aedes aegypti*. It was found what was interpreted as a linear decrease of fecundity with an increasing dose of insecticide (**Fig. 4a**) (61). However, a closer look to the results suggests an alternative interpretation: the number of eggs laid appear to first increase at the lower dose tested (0.025 ppm) before it decreases when the dose increases. The shape of such a dose-response relationship is a typical prediction of a hormetic response: a positive response at low concentration followed by a negative response at high concentration. For comparison, an example of hormesis in a different context is shown in **Fig. 4b**, where a low intensity of gamma radiations was found to increase the fecundity of the house cricket *Acheta domestica*, while high intensities of exposure had the opposite effect (70).

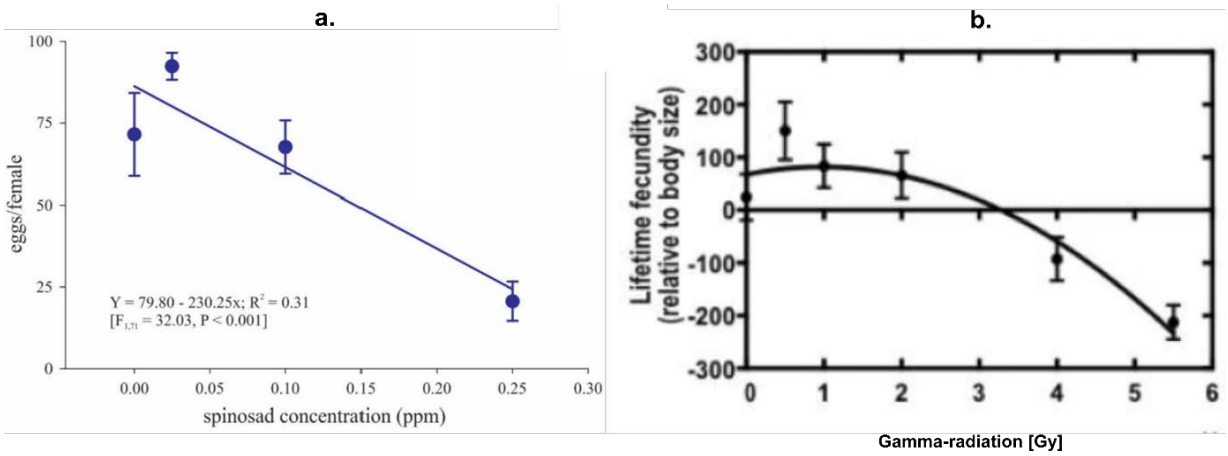

Figure 4. (a) Number of eggs laid by Aedes aegypti mosquitoes exposed at larval stage to various concentrations of the insecticide spinosad. Reproduced with permission from (61). (b) Lifetime fecundity of the house cricket *Acheta domestica* exposed to increasing dose of gamma-radiations. Adapted from (70).

It is however worth to note that, as hormesis is considered to be based on compensatory or repairing mechanisms that may be energetically costly, the extent to which this phenomenon may be observed is likely to be dependent on the environmental conditions and resource availability (71).

Unfortunately, that sublethal and partially lethal doses can, in some cases, lead to drastically different outcomes is often overlooked in the field of vector-borne diseases. Since these effects may ultimately

affect disease transmission or resistance evolution, there is an urgent need for further investigations to take this phenomenon into account.

In the next section, I describe how changes in the life-history of a mosquito vector can be related to its ability to transmit pathogens, with a particular focus on the malaria system.

1.1.4 Unravel mosquito vectorial capacity

To understand how mosquito life-history determines its capacity to transmit an infectious agent, the quantitative components underpinning the dynamic of transmission are usually integrated in the concept of *vectorial capacity* (VC). A broad definition of vectorial capacity being: "the overall ability of a vector species in a given location at a specific time to transmit a pathogen." (72). What is important in this definition of VC is that it not only focuses on the capacity of a vector to biologically acquire, carry and transmit a pathogen, but also includes parameters related to the population dynamics of this vector species. It thus also includes fitness traits such fecundity and longevity. Vectorial capacity has been mathematically described by the Ross-MacDonald model (73) as follows:

$$VC = \frac{ma^2bp^n}{-ln(p)}$$

Where *VC* is vectorial capacity, *m* is the mean number of mosquitoes per host, *a* is mosquito's biting rate, *b* is the probability of transmission from mosquito to host, *p* is mosquito's daily survival, and *n* is the parasite extrinsic incubation period. Combining these parameters results in a value that represents the number of infectious bites arising from one single infectious person for one day, also called *reproductive number* (74). This equation allows for predictions on how mosquito traits contribute to the transmission of a disease. For example, as the extrinsic incubation period of the malaria parasite is long (8-16 days) compared to the life-span of its mosquito host (10-21 days), it has been predicted that a reduction of ca. 8% of mosquito daily survival may lead to a decrease of nearly 80% of its vectorial capacity (75). That a reduction in longevity affects malaria transmission has been observed in Indonesia, where malaria was markedly lower in an area where the mosquito vectors were found to have a reduced life span (76).

The set of parameters of VC that relates to how the mosquito interacts with the parasite is usually called *vector competence* (72). It encompasses mosquito traits associated to its ability to acquire the pathogen (e.g. host-seeking and blood-feeding behavior), to respond to the infection and carry the parasite (i.e. mainly the immune response (77, 78)), and to transmit the pathogen (e.g. biting behavior). In the equation, vector competence is involved in mosquitoes' biting rate *(a)*, but mainly determines the probability that a mosquito bite is actually infectious *(b)*.

Vectorial capacity, by relying on several biological and fitness parameters, is often found to be dependent on the environmental conditions in which the vector species grow and live (reviewed in (79) for the malaria system). Main environmental factors affecting the ability of mosquito to transmit pathogens include temperature (80–83), food availability (84–89), exposure to xenobiotic (90–95), and the presence of predators (96) or other pathogens (97–99). The sensitivity of VC components to environmental conditions stress the importance of considering the ecology of vector in the epidemiology of vector-borne diseases.

1.1.5 Resistance and vectorial capacity

Sublethal doses may also contribute to the evolution of resistance, providing they affect mosquitoes' fitness in some inheritable way. They can also do so if they induce a hormetic response: instead of decreasing the fitness of the least resistant individuals, insecticide at sublethal concentration may increase the fitness of the most resistant ones. So far, this possibility has received little attention (100). Further, after it has evolved, resistance may also be considered as a parameter of vectorial capacity if 1) if resistance directly affects the ability of mosquitoes to acquire, carry, and transmit the pathogen, or 2) if resistance impedes the efficacy of vector control tools. In the first case, vectorial capacity may be affected by resistance because of the fitness costs it often imposes to mosquitoes (101–104). The size of the effect is likely to be condition-dependent, since the costs of resistance are sensitive to environmental perturbations such as high larval density (105), the presence of pathogens (106, 107), or the presence of xenobiotic against which the resistance is ineffective (108).

In the second case, insecticide resistance may increase mosquitoes' vectorial capacity if it allows the mosquito to bypass vector control tools as IRS or ITNs. While several studies support this view (109–114), there are little evidences for a significant effect of resistance on malaria transmission at large scale (115, 116).

The need for more works to evaluate this latter possibility is generally acknowledged (117, 118), especially to better understand the observed contrast between the results of entomological and epidemiological studies (119).

1.2 Thesis introduction

1.2.1 Research aims

The research presented in this thesis aims to assess the impact of a pyrethroid insecticide (permethrin) at sublethal dose on the biology of the malaria vector *Anopheles gambiae*. Virtually, any effect of the toxic compound on mosquito's life-history traits is likely to affect its vectorial capacity and may contribute to the evolution of insecticide resistance. Therefore, an emphasis has been put on these two aspects. Mosquitoes being particularly likely to be exposed to insecticides in their larval

environment, the focus was primarily put on the carry-over effects induced by an early-life insecticide exposure. In malaria-endemic areas, mosquitoes may additionally be exposed at adult stage, where ITNs or IRS are used against them. Hence, in two out of the six experiments presented (Chapters 3 and 5), mosquitoes were exposed at both stages. Finally, the effect of larval competition for food was also studied in combination with the insecticidal stress (Chapters 3-5). In nature, resources are often limited and impose an additional constraint to the developing larvae. The obtained results may have both epidemiological and evolutionary implications for malaria control and insecticide resistance, and help to understand how organisms live and adapt in insecticide-polluted environments.

The different experiments (chapters) are structured as follows: we first tested the consequences of permethrin at sublethal dose on mosquito's main life-history traits (Chap. 2), and on mosquito competence for the malaria parasite (Chap. 3). As the insecticide was found to affect malaria prevalence, we tested whether permethrin exposure affected mosquito's immune defence (Chap. 4). Further, we assessed the effects of insecticide exposure on oxidative stress as a possible physiological mechanism through which immunity and other mosquito traits could be affected (Chap. 5). Finally, in the last two last chapters, we used a next-generation insecticide-treated bed-net (ITN) to test the capacity of mosquitoes to bite through it (Chap. 6), and tested whether a sublethal dose of insecticide and/or a blood meal through an ITN may ultimately lead to the evolution of resistance (Chap. 7). In the following sections, the different chapters are briefly introduced.

1.2.2 Effects of a sublethal exposure at larval stage on mosquito fitness

Sublethal dose of insecticides at larval stage may affect the fitness of mosquitoes either by direct poisoning or by inducing indirect carry-over effect. While several studies using partially lethal doses of insecticides found detrimental effects on behavior (44) and fitness-related traits (41, 53, 55), recent advances in eco-toxicology suggest that sublethal doses may, on the opposite, lead to a higher fitness (69, 120). In this chapter, we tested these two contradictory predictions by exposing mosquito larvae to a sublethal dose of insecticide and measuring mosquitoes' development, fecundity and longevity. As the consequences of an initial exposure may be transferred or observed on the next generation (63), we also extended the initial question by assessing the fitness consequences of the insecticide on two successive generations. We also studied how maternal environment and genetic background affect the progeny's response to insecticide by using half-sib families and comparing the performance of the offspring according to the identity of their mother. Finally, we tested whether a sublethal exposure at larval stage has an impact on the tolerance of adult mosquitoes to the same compound. Several studies done with different compounds indeed suggest that tolerance may be plastically regulated (121–123). This study is a necessary first step to predict both short and long-term impacts

of insecticide at sublethal concentration on mosquitoes' life-history, resistance evolution, and, to some extent, malaria transmission.

1.2.3 Low dose of insecticide and mosquito competence for malaria

Mosquito larvae exposed to an insecticide may suffer from long-term physiological consequences related to the detoxification of the toxic compounds (124–126), and/or to the associated costs (101, 127). Later in life, these physiological changes may affect the way mosquitoes respond to an infection by the malaria parasite. Adult mosquitoes exposed to deltamethrin (PY) were, for instance, found to have a decreased prevalence and intensity of infection with *Plasmodium* (128). In addition, pyrethroid insecticides are also known to have a direct effect on *Plasmodium* development (129). In this chapter, we tested whether similar effects can be observed when insecticidal stress is applied at sublethal concentration and at different life stages by exposing mosquitoes as larvae, adults, or both. In addition, we included larval competition to assess the importance of larval nutrition in mosquitoes' responses to insecticidal stress. Resource availability is, a major determinant of mosquito vectorial capacity (86, 88, 130, 131), but its interaction with insecticidal stress has received less attention (95, 132). Mosquitoes were tested for their survival after they received an infectious blood meal, the prevalence of infection and their infectiousness at the end of the parasite's incubation period. This experiment helps to understand how malaria transmission may be affected by the presence of pyrethroid residues in mosquito's larval and adult environments.

1.2.4 Low dose of insecticide and mosquito immune responses

Insecticides can affect the immunity of mosquitoes in various ways (57). For example, insecticides can affect the number of hemocytes (133, 134), which are involved in phagocytosis, nodulation and encapsulation (135). Also, the enzyme phenoloxidase, which is involved in immune melanogenesis (136), was found to be possibly associated insecticide resistance (137, 138), suggesting a link with detoxification. It is however unclear whether a sublethal exposure at larval stage affects the immune responses of adult mosquitoes. Here, we tested this idea by exposing mosquito larvae to a sublethal dose of insecticide, and assessed the immune responses of adult mosquitoes in two ways. First, we measured the melanization response of mosquitoes, which is an important mechanism of immunity (135, 139) that is also used against the malaria parasite (140, 141). Secondly, we measured the capacity of adult mosquitoes to suppress a bacterial infection. The antibacterial response results from the combined effects of several aspects of immunity, including phagocytosis, the use of reactive compounds, and melanization response, all of which being also used against *Plasmodium* parasites (135, 142). In addition, we tested the effect of larval competition for food to assess whether the long-term effects induced by the insecticide on immunity is dependent on larval nutrition.

1.2.5 Low dose of insecticide, mosquito oxidative state and melanization response

The balance between pro-oxidants and antioxidants is involved in crucial biological functions such as reproduction (143–145), longevity (146, 147), and immunity (148, 149). To defend themselves against parasites, mosquitoes use reactive oxygen species (ROS) as a direct weapon to kill pathogens (150–153), but also as cell signalling molecules (148) or to trigger the melanization biochemical cascade (154). As pyrethroid insecticides are known be a major source of oxidative stress (OS) in exposed organisms (155), the ability of insects to re-establish their oxidative balance (i.e. their antioxidant capacity) is likely to play a key role on the physiological consequences of such exposure. In this chapter, we tried to understand how both larval exposure to insecticide and competition for food modulate the oxidative state of adult mosquitoes, and how differences in their antioxidant capacity affect their melanization response. Further, as it was found in *Drosophila melanogaster* that a repeated stress may increase the effect on OS of an initial stress (156), we tested how a second exposure at adult stage interacts with both larval exposure and larval competition to affect mosquito's oxidative balance. These measurements are important as they allow to better understand the mechanisms underlying several of the phenotypic changes induced by the exposure to an insecticide.

1.2.6 Taking a blood meal through an ITN, survive and lay eggs

Insecticide-treated bed-nets offer an effective physical and chemical protection against infectious mosquito bites (19, 157). The pyrethroid insecticides used on these nets are strongly irritant for mosquitoes (38, 158), keeping them from staying on the net for too long. To bite a person sleeping under the net, mosquitoes either have to find a hole in it, or bite directly through the net if its user is touching it. In this chapter, we tested this possibility using a next-generation ITN (Olyset Plus® net) that is treated with permethrin and a synergist (piperonyl butoxide – PBO) that slows down its detoxification. As mosquitoes may suffer from the irritant and toxic effect of these chemicals when trying to bite, we also recorded the biting and feeding behavior of mosquitoes, and measured the long-term fitness consequences on mosquitoes that succeeded to take blood through the net. This study contributes to fill the gap in the current knowledge on the efficacy of ITNs. Indeed, the possibility for mosquitoes to take a blood meal through an ITN is only indirectly measured in standard procedures (e.g. (159)), and the possibility for mosquitoes to bite without taking a blood meal (e.g. if they are too irritated to take blood) is usually overlooked. Similarly, the consequences of such blood meal on mosquitoes' fitness is rarely studied (but see (160, 161) for studies with resistant mosquitoes), but help us understand the long-term effect of an exposure to an ITN on both the risk of malaria transmission and the selection for resistance.

1.2.7 Low dose of insecticide, ITN and the evolution of resistance

Insecticide residues spread in the environment by agricultural practice is considered as the main selection pressure acting on mosquitoes for the evolution of resistance (162–165). However, results from field studies suggest that ITNs may also contribute significantly to the evolution of insecticide resistance in mosquito vectors (166–168). In this chapter, we tested this possibility by selecting adult mosquitoes on an Olyset Plus® net during 11 generations: for mosquitoes to transmit their genes to the next generation, they had no choice but biting through the ITN and survive long enough to lay eggs. In addition, we tested whether a sublethal exposure alone or in combination with the selection on adults could contribute to the evolution of resistance. A previous study already tested the effect of a pre-exposure to a pyrethroid on a further selection on mosquito larvae, and while they could not select for significant resistance, they found that gene expression was differentially selected whether larvae were sublethally exposed or not prior to the selection (169). Our study is, to our knowledge, the first to test the selection potential of an ITN containing PBO, and that experimentally assesses the possibility for an entirely sublethal dose to select for insecticide resistance. Finally, the selected mosquitoes were tested for their ability to bite through the ITN, survive and lay eggs to evaluate how resistance can modify their capacity to do so. This study provides new evidences on the conditions that favour the evolution of resistance, and on how resistance can affect – or not – the protection granted by PBO-based ITNs.

1.3 Experimental system

1.3.1 The mosquito: *Anopheles gambiae*

In all the experiments presented in this thesis, we used the insecticide-sensitive Kisumu strain of the species *Anopheles gambiae* (166). This species is one the most important vector of malaria in sub-Saharan Africa (170) and is also a vector of lymphatic filariasis (171) and O'nyong-nyong virus (172). The strong vectorial capacity of this mosquito is partially explained by its host preference and activity pattern: *An. gambiae* females being most active at night inside the dwellings (endophilic) and bite essentially on humans (anthropophilic) [2]. Before mosquitoes reach adulthood and become potential vectors, they go through a complex life cycle that includes four distinct stages, three in water and the last one being aerial: egg, larva, pupae and adult (**Fig. 5**). At adult stage, mosquito females can acquire and eventually transmit parasites by taking a blood-meal on their host, which is an essential source of proteins for them to produce their eggs (173). An adult usually lives between 10-21 days (4), and one gonotrophic cycle take 2-4 days (174). Thus, a single female is expected to complete 5-10 gonotrophic cycles during its life. After a blood meal, mosquito females show little preference for their breeding site and can lay eggs in nearly every spot of water available (except fast running water, muddy or shaded water) (175). Therefore, eggs of *An. gambiae* are likely to be found in polluted water spots of

agricultural or urban areas, exposing freshly hatched larvae to various xenobiotic, including insecticides.

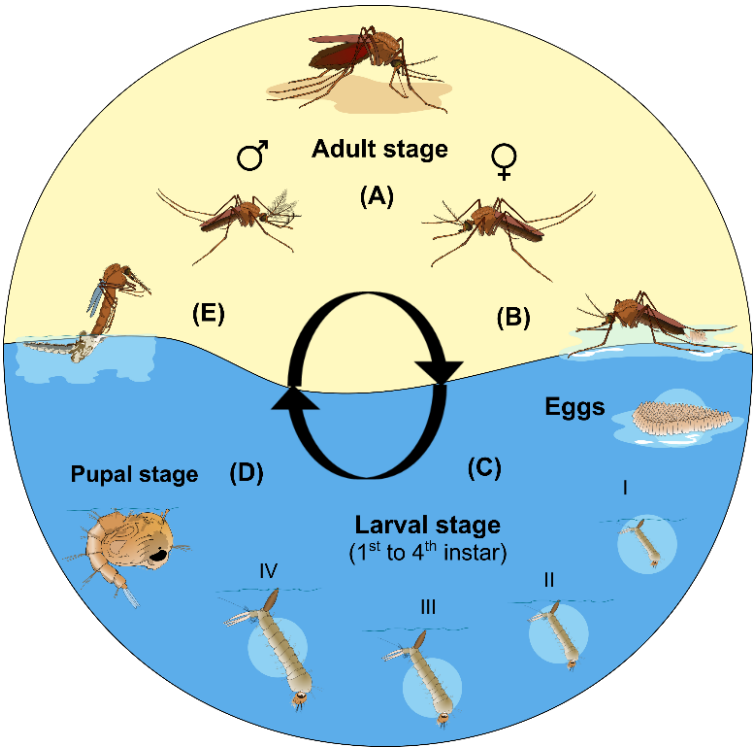

Figure 5. Mosquito life cycle. Adult females of most mosquito speacies are anautogenous and must take a blood meal to produce eggs (A). The eggs are laid at the surface of water (50-200 eggs per female) (B). Resulting larvae grow in the aquatic environment and go through 4 successive instars (C) before they pupate (D). At pupal stage, mosquitoes are unable to feed and start their metamorphosis to become adults. After 2 to 4 days, adults emerge from the pupae (E).

In our lab, a colony of about 1200 individuals is maintained at 26.5 ± 0.5 °C and 70 ± 5% humidity, with a 12:12 photoperiod. Adults have constant access to a 6% sucrose solution. Larvae are reared by groups of 150 in trays containing 800 mL of deionized water and fed with Tetramin® Baby fish food. Adult females are blood-fed fed once per week on a human arm in order to maintain their anthropophilic nature. Using this strain is particularly relevant in experiments where host-seeking or feeding behavior on a human host are assessed (Chapters 6 and 7).

1.3.2 The insecticide: permethrin (pyrethroid)

Pyrethroids are neurotoxic insecticides derived from pyrethrum, a 150 years old botanic insecticide found in *Chrysanthemum* spp. (176). They act on the peripheral and central nervous system by keeping the voltage-gated sodium channels open, leading to repeated neuron firing and overexcitability (30) (**Fig. 2**). To reach mosquitoes' nervous system, the insecticide penetrates directly through the cuticle by simple contact (177) (**Fig. 3**).

Pyrethroid insecticides are widely used for the control of vector-borne diseases including malaria, dengue, Chagas disease, and leishmaniasis: a large scale study found that pyrethroids were the first

class of insecticide used against vector-borne diseases in terms of the area covered (178). For malaria control, pyrethroids are used for indoor residual spraying (IRS) and are the only class of insecticides used on insecticide-treated bed-nets (ITNs) because of their low toxicity to humans (179).

Permethrin has been chosen as a pyrethroid model as it is currently used on a next-generation ITN (Olyset Plus®), but also in agriculture, forestry, horticulture, as well as for various insecticidal products made for private uses (180, 181). Unsurprisingly, permethrin residues have been found in fresh water (182) and sediments (183) at a global scale, thereby increasing the risk for mosquitoes to be exposed to this compound (or related compounds) during their development.

Permethrin exists in two isomeric forms, trans- and cis- permethrin (**Fig. 6**), the cis- being usually found to be more toxic than the trans- isomer (184, 185).

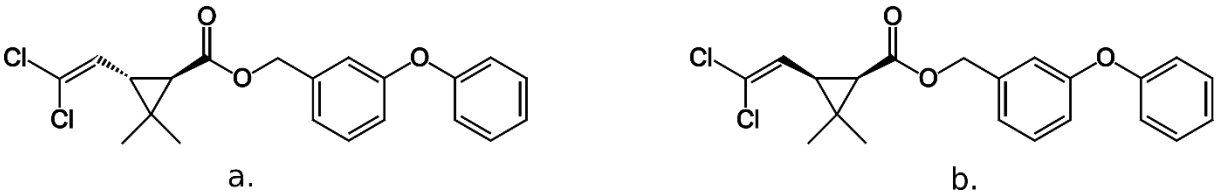

Figure 6. Molecular structure of (a.) trans- and (b.) cis- permethrin.

Permethrin formulations for vector control or agriculture are made with a mix of cis:trans isomers at various ratios (usually 40:60, sometimes 80:20 or 25:75). In our experiments, we used permethrin at analytical grade (≥95% pure) (Sigma-Aldrich, St.-Louis, Missouri) containing a mixture of cist- and trans-isomers at ca. 40:60 ratio. The permethrin-treated Olyset Plus® net, which was used in two experiments included in this thesis (Chapters 6 and 7), uses the same 40:60 cis:trans ratio (186).

Permethrin has a low solubility in water (0.2 mg/L) and a high adsorption on plastic material (187). For its use in our experiments, the insecticide was therefore first dissolved in ethanol, and glass material was used instead of plastic one to rear mosquito larvae. Permethrin and pyrethroids in general have a negative temperature coefficient, meaning that their toxicity increase when temperature decreases (188, 189). To prevent any bias, all the manipulations involving the exposure of mosquito larvae or adults to permethrin were done at 26.5 °C.

Chapter 2

Effects of permethrin at sublethal concentration on the main fitness traits of the malaria vector *Anopheles gambiae*

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2.1 Abstract

During their development, mosquito larvae may be exposed to various xenobiotics, including insecticides. Insecticide residues are frequently found in fresh water worldwide, and can have long-term consequences on mosquitoes' life-history and ability to transmit pathogens. Until now, research in this field has mainly focused on the effects of semi-lethal concentrations. However, recent studies suggest that entirely sublethal concentrations may lead to different and sometimes opposite outcomes. Using the malaria vector *Anopheles gambiae*, we tested this idea by measuring the consequences of a strictly sublethal concentration of the pyrethroid insecticide permethrin on mosquito's fitness over two generations. Egg-hatchability, larval development, fecundity, longevity, and adult tolerance to permethrin were measured after mosquito larvae were reared with or without the insecticide. We found that in the initial generation, females reared with the insecticide developed slower but tended to have a higher egg-laying success than their unexposed counterparts. This beneficial effect of permethrin was however found to come at a cost, as the longevity of exposed mosquitoes' progeny was reduced. In addition, we found that egg-hatchability was not affected by the presence of insecticide in the water or the exposure of the mothers. Finally, we confirm that a pre-exposure to permethrin leads to a higher tolerance to the insecticide later in life. Together, our results suggest that insecticide at sublethal concentration likely has both detrimental and beneficial consequences the fitness of mosquitoes, which may directly impact their vectorial capacity and the evolution of resistance.

2.2 Introduction

Insecticides are one of the most effective tools to fight malaria (18, 19). They are primarily used in indoor residual spraying (IRS) and on insecticide-treated bed nets (ITNs) to prevent infectious mosquito bites. In addition to vector control, insecticides are also widely used in conventional agriculture to protect crops from pest insects, contributing to the spread of insecticide residues in fresh water (27, 28). This situation has created conducive conditions to the evolution of insecticide resistance in mosquitoes, which is now widespread in malaria-endemic countries (190). While lethal doses are likely to be the main causative agent of resistance, several studies showed that insecticides at low concentration are also likely to affect important life-history traits as egg-hatching, adult size, fecundity or longevity (53, 55, 191, 192).

Further, very low doses of insecticide may also trigger a stimulatory – or hormetic – response of the organism. Hormesis is the biphasic dose-response observed when environmental stressors induce a beneficial effect at low dose while being toxic at high concentration (65). This phenomenon has already been documented in insecticide-exposed insects for which various beneficial effects were noticed, including decreased development time, increased body length, enhanced fecundity or longer life span (reviewed in (69)). A plastic increase of larval tolerance to insecticides was also reported in mosquito larvae after they were first exposed to different xenobiotics (124, 193, 121, 123). However, hormesis itself is expected to be energetically costly, and may therefore be involved in physiological trade-offs (71). For example, a study found that after a sublethal exposure to a neonicotinoid insecticide, reproduction of the green peach aphid increased during the first generation, but the offspring suffered from a decreased reproductive success and longevity (63).

By affecting insects' fitness, insecticide at sublethal concentration may favor the evolution of resistance, and, when applied to a vector species, affect its overall ability to transmit pathogens (i.e., its vectorial capacity (72)). In the case of malaria, a reduced longevity of the mosquito may not allow the *Plasmodium* parasite to reach the infectious stage, which impedes the transmission of the pathogen (76).

If the impact of low doses of insecticides on the evolution of resistance and vectorial capacity of mosquitoes has received some attention, most studies use partially lethal concentrations and/or expose mosquitoes for a limited period of time (60, 123, 191, 194, 195). While this approach has proven to be useful, the observed consequences following an exposure are a combination of the direct selection by the insecticide and the sublethal effect it may induce. Moreover, using high doses may not allow to detect for potential hormetic responses.

To fill this gap and better understand how entirely sublethal concentration of insecticides affect mosquito vectors, we ran two experiments using the malaria vector *Anopheles gambiae* and the widely used pyrethroid insecticide permethrin. First, we tested whether an early-life exposure to permethrin

affected larval development and adult main fitness traits over two successive generations. We also tested whether the effect of permethrin differed according mosquitoes' genetic background using half-sib families. Second, we tested whether an entirely sublethal concentration of permethrin could affect the tolerance of adults to the same insecticide. Transstadial increase in permethrin tolerance was already reported after larvae were exposed to partially lethal concentrations of soap or hydrogen peroxide (122). However, it is currently unclear whether a sublethal dose would lead to similar results.

2.3 Material and Methods

In a first experiment, we used the insecticide-sensitive *Anopheles gambiae s.s* Kisumu strain (166) to assess the effects of permethrin at sublethal concentration on larval development, adult fecundity, and longevity over two generations (hereafter: *fitness experiment*). The hatchability of eggs laid by exposed mosquitoes was also assessed both in water or in a permethrin solution. In a second experiment, we tested the effect of larval exposure to a sublethal concentration of permethrin on adult tolerance to the same insecticide (hereafter: *adult tolerance experiment*).

All experiments were run in an insectary maintained at 26.5 ± 0.5 °C, 70 ± 5% humidity and a 12:12 light to dark photoperiod.

2.3.1 Determination of the sublethal dose of permethrin

To determine the concentration of permethrin to be used in the main experiments, we tested the effects of 3 concentrations (0.1, 0.15 and 0.2 µg/L) on the mortality of individually reared *Anopheles gambiae s.s.* larvae (40 larvae per concentration). Solid permethrin (Sigma-Aldrich Inc., St. Louis, Missouri) was first dissolved in pure ethanol to obtain a 1 µg/mL solution. This solution was then diluted in the adequate volume of deionized water to reach the desired concentrations. For the control, a solution of 0.015% volume per volume (118 µg/L) ethanol was used. Freshly hatched larvae were then placed individually in glass petri dishes (4 cm diameter x 1.2 cm height) containing 4 mL of solution, and fed according to the regime given below. We recorded mortality as the proportion of adults emerging at each concentration. Ethanol alone (control solution) was not found to induce significant mortality (3/40 (7.5% (95% CI: 2.6 to 19.8%)); tested against a mortality of 0/40 using a 2-sample test for equality of proportions (*prop.test* in R)). The highest concentration of permethrin giving no significant mortality (1/40 (2.5% (0.4 to 12.9%)) was 0.15 µg/L. Permethrin concentration in the following experiment was therefore fixed at 0.15 µg/L, and ethanol concentration (for both control and permethrin exposed larvae) was kept at 0.015% v/v (118 µg/L).

2.3.2 Mosquito rearing and maintenance

Freshly hatched *An. gambiae* larvae (0-3h old) were put individually in glass petri dishes containing 4 mL of deionized water and 118 µg/L (0.015% v/v) ethanol supplemented with permethrin (final

concentration: 0.15 µg/L) or no permethrin. Larvae were provided daily with Tetramin Baby® fish food according to their age: 0.04, 0.06, 0.08, 0.16, 0.32 and 0.6 mg/larva for age 0, 1, 2, 3, 4, and 5+ respectively (196). At the end of larval development, pupae were collected and transferred in 21 x 21 x 21 cm plastic cages according to their larval treatment. Adult mosquitoes had constant access to a 10% sucrose solution that was replaced every 5 days.

2.3.3 Fitness experiment

Fecundity and longevity were measured over two successive generations. Fecundity was assessed at three time points during the first (F0) generation and at one time point during for the F1 generation. We used half-sib families in the first generation to test whether the response of mosquitoes to the insecticide has a genetic basis.

half-sib families

To build the half-sib families, 60 freshly hatched *Anopheles gambiae* larvae from our lab colony were haphazardly chosen and reared individually in well plates with controlled food amount as described above. After emergence, males and females were placed in a cage for 72h to allow males to fertilize the females. Females were then blood fed on a human arm for 8 min, and fully engorged females were transferred 24 hours later in individual 150mL cups that contained water for mosquitoes to lay their eggs. The batch of eggs laid by one single female constituted one half-sib family, and the eggs from 10 haphazardly chosen females were used to build the F0 generation.

Note that the females used to build the half-sib families were reared individually with controlled level of food to ensure that differences among families in the experiments would be mainly caused by differences in genetic background, and less by maternal effects.

F0 – Larval rearing

From each of the 10 half-sib families, 35 larvae were reared in a permethrin solution (*exposed*), and 35 in a control solution (*unexposed*), for a total of 700 larvae. Pupae were transferred in 21 x 21 x 21 cm plastic cages according to their family and treatment. Males were removed from the cages within the first 24 hours following their emergence to prevent any fertilization with the females to be used in the experiment. They were replaced by at least 25-30 males (per cage) from our *An. gambiae* colony, and given 4 days to fertilize mosquito females. This manipulation allowed us to ensure that any effect on female reproductive fitness was not caused by an effect of permethrin on males.

F0 - Blood meal, fecundity and longevity

Females were blood fed for 8 minutes on GH's arm when they were 5-6 days old. 24 hours after the blood meal, fully engorged females were individually transferred in 120 mL plastic cups covered with a net and containing water in the bottom. Sugar was provided by putting cotton soaked in 10% sugar solution, which was replaced every fifth day. A conical filter paper was placed in the water. Egg-laying

was checked every day for 6 days starting from the blood meal. Once a female had laid its eggs, the filet paper was removed a picture of the eggs was taken. After 6 days, the water from all cups was removed to prevent death by drowning. For the second and third blood meals (at age 18-19 and 26-27 days respectively), females were blood-fed individually on a human arm through the net covering the cups for 8 min.

F0 - Egg ***hatchability***

After the first lay, eggs were collected 4 days after the blood meal from 10 haphazardly chosen females (5 exposed and 5 unexposed females). The eggs laid by each female were divided in two groups and were put in 15 cm diameter glass petri dishes containing either 30 mL of deionized water or a highly concentrated permethrin solution (50 µg/L). Altogether, the 10 females laid 1148 eggs, out of which 565 were immersed in the permethrin solution, and 583 in deionized water. Food was provided once according to the number of eggs. Eggs that successfully hatched were finally counted, and the larvae were removed every day for three days. After this period, eggs that did not hatch were considered dead.

F1 – rearing of larvae

Eggs from the third gonotrophic cycle of the parental generation (F0) were pooled together according to the treatment of the mothers (exposed or unexposed), and resulting larvae were reared individually in ethanol 0.015 % with or without permethrin (final concentration: 0.15 µg/L), in a full factorial design. The identity of the half-sib family was no kept because too few females of each family were still alive and laid eggs after the third blood meal. In total, 480 larvae were reared (120 per treatment). The same protocol as for the F0 generation was followed until females were blood fed.

F1 – Blood meal, fecundity and longevity

Females were given the possibility to blood feed on human arm when they were 3-4 days old. The same protocol as for F0 was followed to collect and count the eggs, but one single gonotrophic cycle was tested in F1. Importantly, sugar was removed at day 13 after the blood meal to test whether nutritional stress could increase or reveal costs induced by the treatments. Mortality was checked daily.

F0 & F1 - Body size

Wing length was used as proxy for body size and weight (197). Wings from dead mosquitoes were dissected and the distance from the axillary incision to the tip of the wing was measured using the software ImageJ v1.51 (198).

2.3.4 Adult tolerance experiment

We reared 200 larvae individually in 0.015% v/v ethanol and 220 in 0.015 % ethanol with permethrin (final concentration: 0.15 µg/L). Pupae were transferred in cages to emerge and adults were provided with a 10% sucrose solution.

Adults females (2 – 4 days old) were exposed for 30 min to WHO permethrin impregnated papers (0.75%) in test tubes (199). After exposure, mosquitoes were transferred by replicate in 150 mL plastic cups and provided with cottons soaked in a 10% sugar solution. Mortality was recorded after 24 hours. In total, 14 replicates of 15 females were assayed, 6 replicates of mosquitoes reared in control solution, and 8 replicates of mosquitoes reared with permethrin. Based on a time-mortality curve assessed prior to the experiment with mosquitoes from our colony, a 30 minutes exposure was expected to kill ca. 80% of the females.

2.3.5 Statistical analyzes

All analyses and graphs were done using the R software (version 3.6) (200). Significance was assessed using the Anova function of the *car* library (201). We used a type III anova in the case of a significant interaction, and a type II anova otherwise.

When relevant, significant interactions were further investigated using post-hoc tests with *emmeans* (using Estimated Marginal Means (EMM)) and *pairs* functions of the *emmeans* library in R, with p-values being adjusted using the *mvt* method.

Fitness experiment

Larval development and mortality

As all larvae in F0 or F1 molted into pupae after 7 or 8 days, development time was analyzed using a generalized linear model (GLM) with binomial distribution of errors, where the response variable was the day of pupation. For F0, explanatory variables were larval exposure (exposed or unexposed) and half-sib family (1-10). For F1, explanatory variables were maternal exposure (i.e., whether F0 females were reared with permethrin or not) and larval exposure. Larval mortality was analyzed using GLM with binomial distribution of errors. Models for mortality used the same explanatory variables as for larval development.

Wing size

Adult wing size was analyzed using an ANOVA. For F0, explanatory variables were larval exposure and half-sib family. For F1, explanatory variables were maternal exposure and larval exposure.

Fecundity and laying success

For the first generation, fecundity was analyzed in two different ways. First, we used GLMs with binomial distribution of errors to analyze mosquitoes' egg-laying success for each gonotrophic cycle separately. Only females that survived long enough after a given blood meal to lay eggs (more than 3 days) were included. Explanatory variables were larval exposure and half-sib families, while wing

length was added as a covariate. We adjusted p-values for multiple testing using a Bonferroni correction.

Second, we used a zero-inflated negative binomial mixed-effect model (ZINB-GLMM) to assess the effect of larval exposure on global fecundity. The function *glmmTMB* of the *glmmTMB* library (202) was used to build the model. We used the number of eggs laid by each female as the response variable. Females that did not lay eggs (either because they died before the last gonotrophic cycle or for any other reason) were considered as having laid 0 egg. Thus, response variable represented the global contribution of each mosquito female to the next generation. In the conditional part of the model, larval exposure, gonotrophic cycle and half-sib family were included as explanatory variables. Wing length was added as a covariate, and female ID was set as a random factor. In the zero-inflated part of the model, mosquito longevity and the gonotrophic cycle were set as explanatory variables, wing length was included as a covariate, and female ID was set as a random factor.

For the generation F1, we analyzed fecundity in the two same ways. First, egg-laying success was analyzed using a GLM with binomial distribution of errors. Maternal exposure and larval exposure were included as explanatory variables. Second, we used the *zeroinfl* function of the *pscl* library (203) to build a zero-inflated negative binomial model (ZINB) to test for any difference of the number of eggs laid among the treatments. In the conditional part of the model, maternal exposure and larval exposure were included as explanatory variables, and wing length was set as a covariate. In the zero-inflated part, longevity was put as an explanatory variable and wing length as a covariate.

Egg hatching success

The proportion of larvae that successfully hatched was analyzed using a mixed-effect generalized linear model (GLMM) with binomial distribution of errors, where the ID of the mother was set as a random factor. Explanatory variables were the exposure of the females that laid the eggs (exposed vs. unexposed), and the solution the eggs were immersed in (water or permethrin).

Longevity

We used Cox's proportional hazard models to analyze F0 and F1 adult longevity. In F0, larval exposure to permethrin and half-sib family were included as the explanatory variables. In F1, maternal exposure and larval exposure were included as explanatory variables. In both cases, wing length was included as a covariate. The proportional hazard ratio assumption was tested using the function *cox.zph* from the *survival* library (204).

Adult tolerance experiment

Larval mortality

We used a GLM with binomial distribution of errors where larval mortality was set as the response variable, and larval exposure to permethrin (exposed vs. unexposed) was included as the explanatory variable.

Adult resistance

We used a GLM with binomial distribution of errors to test for differences in mortality 24h after the exposure to permethrin. Mortality in each tube was set as the response variable, and larval exposure was included as the explanatory variable. The model was weighted by the total number of mosquitoes inside each tube. Tube replicates were set as a random factor.

2.4 Results

2.4.1 Fitness experiment

F0 – Larval development

Out of the 350 larvae reared in each treatment, 8 exposed and 5 unexposed larvae died before pupation (χ^2=0.72, df=1, p=0.397). There was no difference between half-sib families (χ^2=0.19, df=9, p=0.661) and no interaction between larval exposure and half-sib families (χ^2=0.23, df=9, p=0.635). Permethrin was found to delay pupation, as 64.1 % (95% CI: 58.9 to 68.9%) of unexposed larvae had completed their development after 7 days, against only 46.8 % (95% CI: 41.6 to 52.1 %) of permethrin exposed mosquitoes (χ^2=15.22, df=1, p<0.001). In addition, these proportions varied between half-sib families (χ^2=23.38, df=9, p=0.005), and there was a significant interaction between larval exposure and half-sib families (χ^2=19.92, df=9, p=0.018).

F0 – wing length

There was no difference in wing length between exposed and unexposed mosquitoes ($F_{1,301}$=0.00, p=0.981) or between families ($F_{9,301}$=0.54, p=0.841), and no interaction between both factors ($F_{9,301}$=0.84, p=0.579)

F0 – fecundity

During the first blood meal, 157 exposed and 159 unexposed females successfully took blood and were kept for the rest of the experiment. Egg laying success was, for gonotrophic cycles 1, 2 and 3: 64.1% (95% CI: 58.6 to 69.2%), 69.1% (95% CI: 62.9 to 74.7%), and 72.9% (95% CI: 64.6 to 79.8%) (**Fig. 1a**). During the second gonotrophic cycle, there was a trend for a higher egg-laying success in exposed mosquitoes than in unexposed ones (**Fig. 1a** and **1c**, **Table 1**). Laying success differed between half-sib families during gonotrophic cycles 1 and 2, and there was an interaction between larval exposure and half-sib families during the first gonotrophic cycle (**Fig. 1b**, **Table 1**).

Table 1. Results of the binomial GLMs for egg-laying success. Given are likelihood-ratio test Chi-squares with their degrees of freedom (χ^2_{df}), and p values adjusted using a Bonferroni correction. Significance is marked with an asterisk.

	First gon. cycle (N=304)		***Second gon. cycle (N=230)***		***Third gon. cycle (N=129)***	
	χ^2_{df}	p	χ^2_{df}	p	χ^2_{df}	p
larval exposure	0.65_1	1	4.59_1	0.096	1.44_1	0.689
half-sib family	25.17_9	0.008*	22.88_9	0.019*	14.77_9	0.293
wing length	0.05_1	1	0.08_1	1	0.63_1	1
larval exposure x half-sib family	22.54_9	0.022*	13.33_9	0.44	3.48_9	1

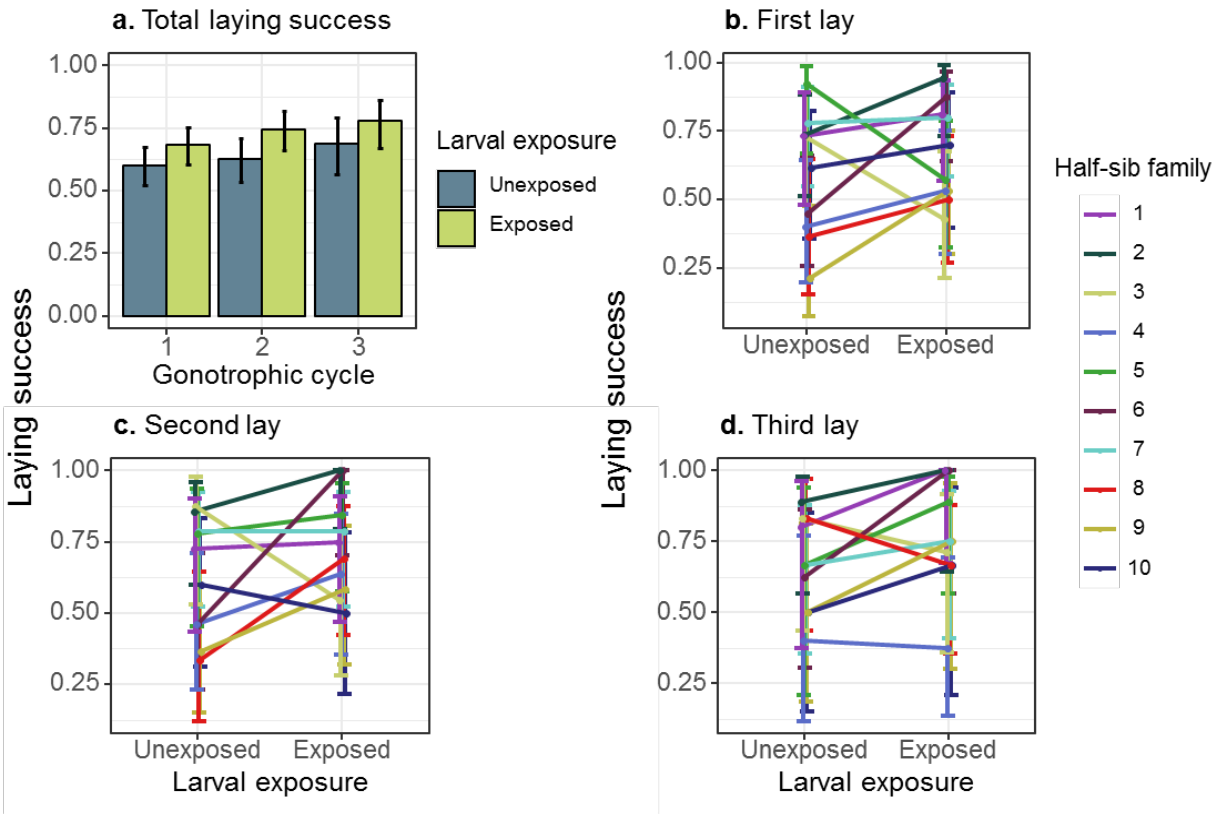

Figure 1. (a): Proportion of females that successfully laid eggs during each gonotrophic cycle according to the exposure of mosquito larvae to permethrin. Other panels show the egg-laying success for each half-sib family (colors) according to larval exposure to permethrin (colored lines connect the family means for each treatment), for gonotrophic cycle 1 (N=304; (a)), 2 (N=230; (b)) and 3 (N=129; (c)) respectively. Error bars show the 95% confidence intervals.

Over the 3 gonotrophic cycles, exposed females laid a total of 20'555 eggs, against 16'613 for unexposed females. The number of eggs laid by female (including those that did not lay eggs (fecundity fixed to 0)), was not found to be affected by larval exposure (χ^2=0.24, df=1, p=0.624, **Fig. 2a**). However, fecundity differed between gonotrophic cycles (χ^2=20.72, df=2, p<0.001), and between half-sib families (χ^2=26.74, df=9, p=0.001). There was also a significant interaction between larval exposure and half-sib families (χ^2=26.91, df=9, p=0.001, **Fig. 2b**), but no interaction between larval exposure and gonotrophic cycle (χ^2=1.78, df=18, p=0.321, **Fig. 2a**), and no triple interaction between larval exposure,

gonotrophic cycle and half-sib family (χ^2=10.64, df=18, p=0.909). Contrast analysis showed that fecundity was the highest during the first gonotrophic cycle, the lowest during the second gonotrophic cycle, and in between during the third gonotrophic cycle (all p<0.001; **Fig. 2a**).

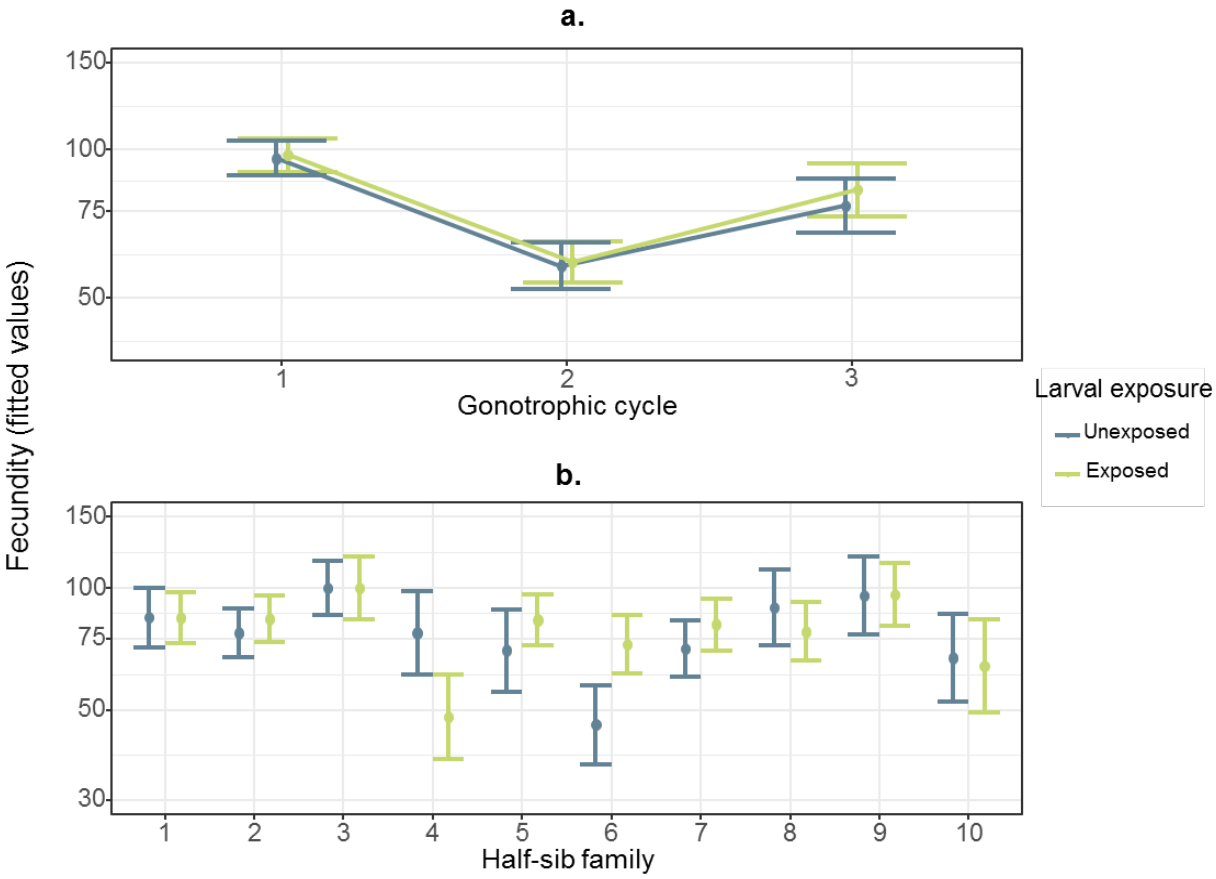

Figure 2. Upper panel (a) shows the fitted values of fecundity (the number of eggs laid per female) for each gonotrophic cycle, according to the solution mosquito females were reared in (larval exposure). N=304, 230 and 129 for gonotrophic cycle 1, 2 and 3. Lower panel (b) shows the fitted values of fecundity for each half-sib family according to larval exposure, irrespective of the gonotrophic cycle. Scale has been log-transformed. N=663. Error bars show the 95% confidence intervals.

F0 – Longevity

F0 females survived from 2 to 52 days after the first blood meal. There was no difference of longevity between exposed and unexposed mosquitoes (χ^2=1.76, df=1, p=0.184) or between half-sib families (χ^2=5.51, df=9, p=0.788), and no interaction between the two factors (χ^2=14.15, df=9, p=0.117).

Egg hatching success

The females chosen for the hatching assay laid between 94 to 152 eggs. Altogether, 592 out of 1148 eggs hatched (51.7% (95% CI: 48.8 to 54.6%). Egg hatchability was not affected by the solution they were immersed in (water or permethrin) (χ^2=2.65, df=1, p=0.104), or by the exposure status of the mothers (χ^2=1.09, df=1, p=0.296). There was no interaction between maternal exposure and egg hatching solution (χ^2=0.748, df=1, p=0.387).

F1 – Larval development

Out of the 480 larvae, 8 died before pupation (1.7% (95% CI: 0.8 to 3.2%)). Mortality was not affected by the exposure status of the mothers (χ^2=0.52, df=1, p=0.473), larval exposure (χ^2=0.52, df=1, p=0.473), and there was no interaction between both factors (χ^2=1.795, df=1, p=0.18). About one third of the larvae finished their development after 7 days (30.3% (95% CI: 26.3 to 34.6%), and this proportion was not affected by the exposure of the mothers (χ^2=0.12, df=1, p=0.724), larval exposure (χ^2=1.079, df=1, p=0.299), or the interaction between both factors (χ^2=0.06, df=1, p=0.815).

F1 – Wing length

The exposure of the mothers did not affect their progeny's size ($F_{1,198}$=0.66, p=0.417). However, F1 mosquitoes exposed to permethrin were marginally bigger than their unexposed counterparts (3.14 ± 0.02 against 3.10 ± 0.02 mm (mean ± 95% CI); $F_{1,187}$=5.69, p=0.018). There was no interaction between both factors ($F_{1,187}$=0.847, p=0.358).

F1 - Fecundity

Between 49 to 52 females of each treatment successfully took blood and were kept for fecundity and longevity measurements. Of the 194 mosquitoes that lived long enough to lay eggs, only 69 did (35.6% (95% CI: 29.2 to 42.5%)). Egg laying success was not affected by maternal exposure (χ^2=0.47, df=1, p=0.492), larval exposure (χ^2=1.00, df=1, p=0.752), or their interaction (χ^2=0.01, df=1, p=914, **Fig. 3a**). The number of eggs laid was not affected by maternal exposure (χ^2=0.83, df=1, p=0.361), larval exposure (χ^2=0.41, df=1, p=0.521) or their interaction (χ^2=1.24, df=1, p=0.265, **Fig. 3b**).

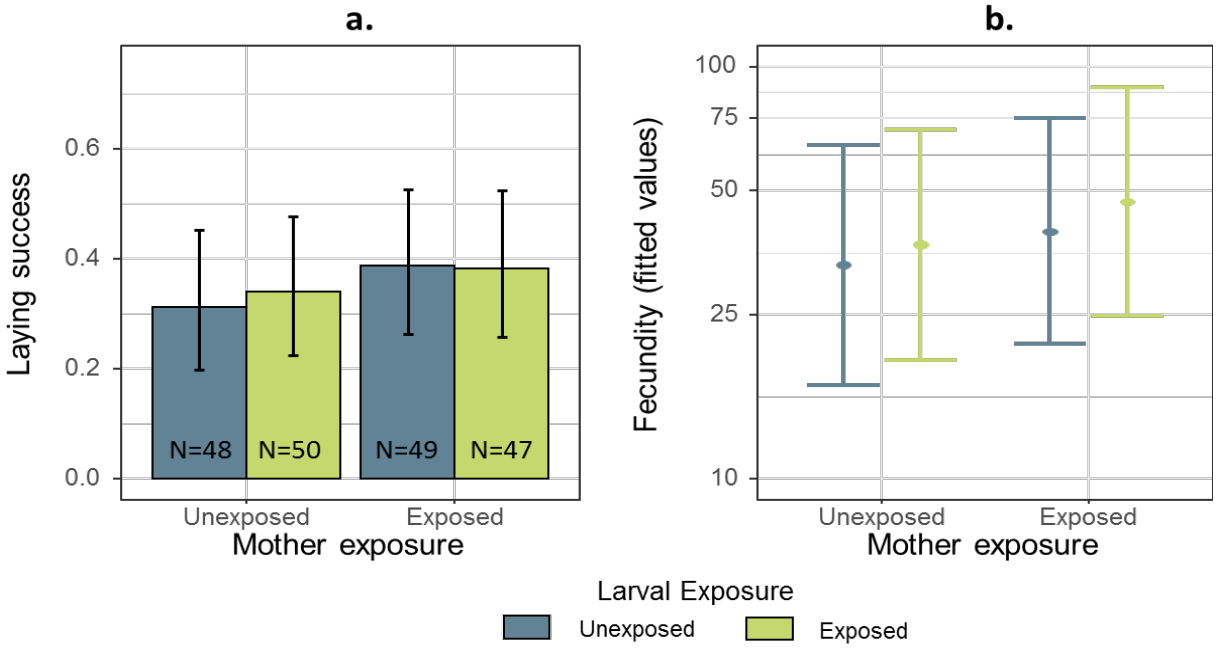

Figure 3. (a) Proportion of fed females that laid eggs (N=194) and (b) fitted values (log-scaled) of the ZINB model of fecundity according to the exposure status of the mothers (F0), and the exposure of F1 mosquitoes at larval stage (colors) (N=69; by treatment (left to right): N=15, N=17, N=19, N=18). Fecundity is the number of eggs laid by mosquitoes, including those that did not lay eggs (fecundity = 0). Error bars show the 95% confidence intervals.

F1 – Longevity

We found a negative effect of the exposure of the mothers on longevity (χ^2=5.41, df=1, p=0.02) but no effect of larval exposure (χ^2=1.56, df=1, p=0.212). In addition, there was a significant interaction between both factors (χ^2=4.99, df=1, p=0.0255, **Fig. 4**): larval exposure had no effect on the longevity of mosquitoes from unexposed mothers (z=-0.90, p=0.366), but there was a trend towards a decreased longevity of exposed mosquitoes from exposed mothers (z=1.93, p=0.053).

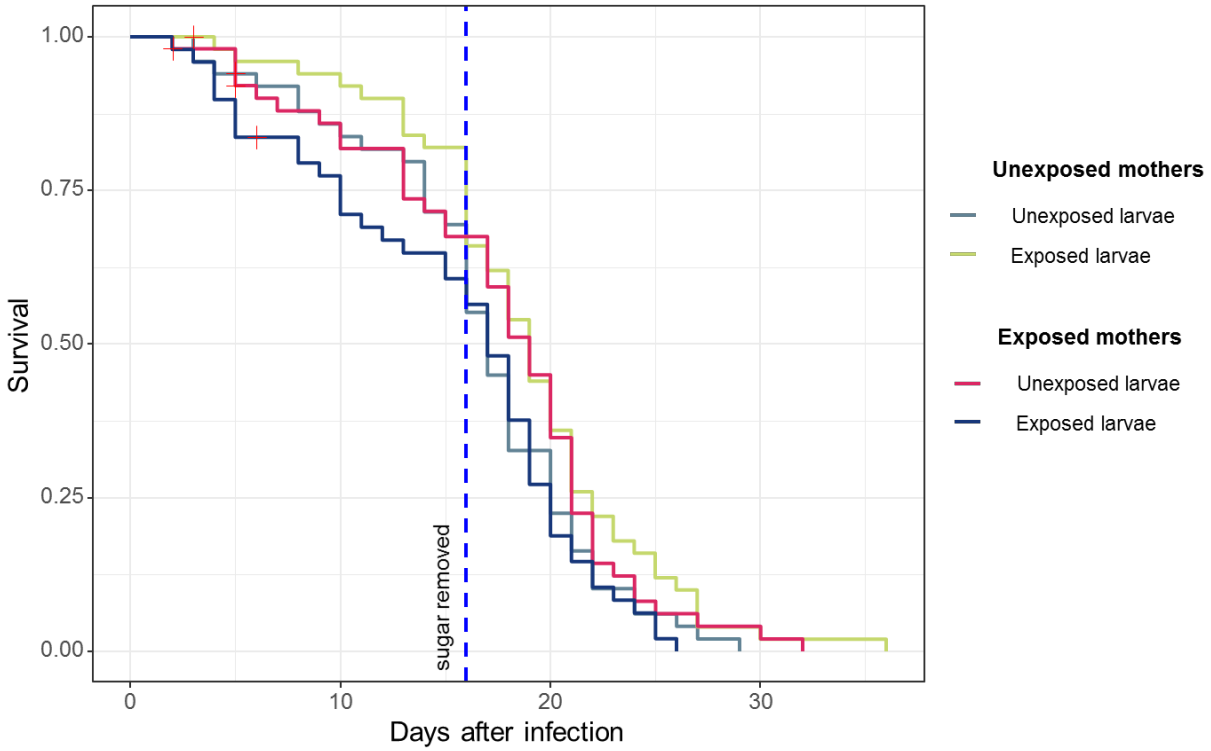

Figure 4. Survival of the F1 generation according their exposure at larval stage and the exposure status of their mothers. In the same order the treatments are presented in the legend: N=46, 47, 52 and 43. The vertical dashed blue line indicates the day from which mosquitoes did not have access to sugar anymore.

2.4.2 Adult tolerance experiment

Larval mortality

4 out of the 220 larvae reared with permethrin (1.8% (95% CI: 0.7 to 4.6%)), and 5 out of the 200 larvae reared in water (2.5% (95% CI: 1.1 to 5.7%)) died before pupation. This difference was not significant (χ^2=0.23, df=1, p=0.630).

Resistance assay

In total, 85 females reared in permethrin and 101 females reared in water were exposed to permethrin impregnated papers. We found that 92.1% (95% CI: from 85.1 to 95.9%)) of the females reared in water, and 77.4% (95% CI: 67.7 to 85.2%)) of the females reared in permethrin died within 24 hours following the exposure. This difference was statistically significant (χ^2=7.17, df=1, p=0.007, **Fig. 5**).

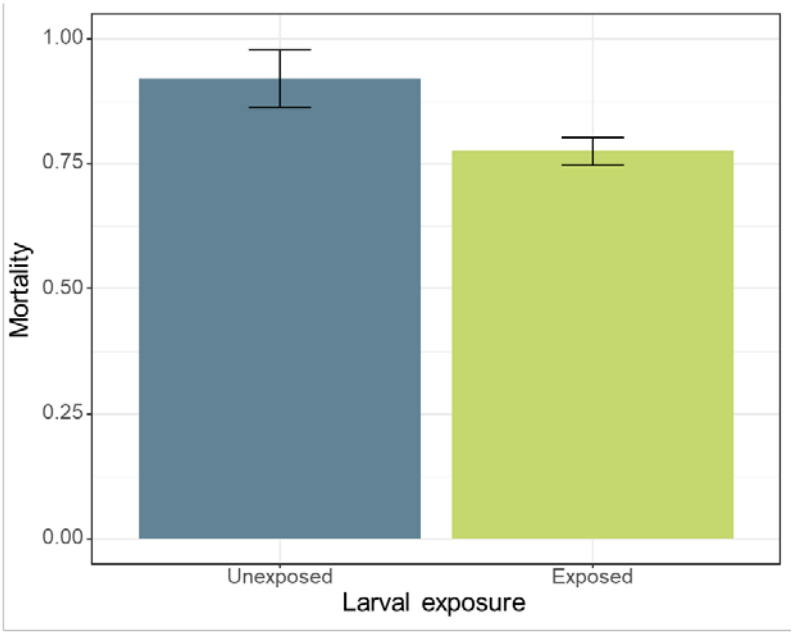

Figure 5. Adult mortality 24h following a 30 minutes exposure to permethrin 0.75% in WHO test tubes. Error bars show the 95% confidence intervals based on replicates (N=8 and N=6 for unexposed and exposed treatment respectively).

2.5 Discussion

We showed in this experiment that a sublethal dose of permethrin can induce both a stimulatory response and impose cross-generational costs to mosquitoes. In particular, we found that reproductive success and adult tolerance to permethrin increased after mosquitoes were exposed at larval stage, but the longevity of their progeny was decreased.

2.5.1 Fitness experiment

F0 larvae exposed to permethrin took more time to develop, suggesting either a direct deleterious effect of the insecticide or the involvement of energetically costly detoxifying mechanism (101). In addition, differences between half-sib families, and their interaction with larval exposure suggest that the identity of the mother – mainly its genetic background, as environment was controlled – played a significant role on the response of its progeny to permethrin. In addition, larval mortality of both generations was low (<3%) and not affected by larval exposure or the exposure of the mothers.

While females from the initial generation were all of similar size, F1 mosquitoes, however, were marginally bigger (+1.2%) when reared in permethrin. A possibility is that exposed larvae had to compensate for the potential costs of insecticide detoxification (101, 127), leading to an increased food consumption and a marginal increase in adult size, e.g. through compensatory growth (205).

Despite there was 2 less females in the permethrin exposed groups of the F0 generation (157 against 159), the total number of eggs that were laid by exposed females over the three gonotrophic cycles was higher by 24%. As we found a trend towards an increase in egg-laying success but no effect of permethrin on the number of eggs laid, this difference is likely explained by the former. Importantly,

this increase in egg-laying success did not trade off with egg hatchability, as neither the exposure of mothers nor the presence of insecticide in water affected egg-hatching success, contrarily to what was found in other studies (206, 207) . Also, while the effect of permethrin on egg-laying success was mainly positive, effect size and direction were dependent on the half-sib family (**Fig. 1**). This is in line with the results on larval development and suggests that the maternal genotype has a strong influence on mosquitoes' response to the insecticide for fitness-related traits. Similarly, the significant interaction between permethrin exposure and half sib family for the number of eggs laid suggests that there may be a potential for evolution to take place, despite the absence of an overall effect of the insecticide on that trait.

The analyses of the F1 generation showed that permethrin exposure of the F0 mothers did not affect their progeny's egg-laying success and fecundity. Hence, there was no obvious cross-generational costs nor synergistic beneficial effect of permethrin on reproductive success. Also, contrarily to what was found in the initial F1 generation, larval exposure did not increase egg-laying success of female. As there was only a trend in the first generation, we cannot exclude that this effect was only observed by chance. Alternatively, the effect in F1 females may have been hidden by their very low egg-laying success (nearly half of that of F0 females). Possibly, the age of the F0 mothers when they laid the eggs to form the second generation (they were fed at 26-27 days old) may have affected their progeny's egg-laying success in some way (e.g. (208, 209)).

Longevity in the first generation was surprisingly stable across larval treatments, with no difference between half-sib families and no interaction with the insecticidal stress. Survival was initially expected to be lower for permethrin exposed individuals because of the energetical cost of detoxification (101). Possibly, females could compensate for the increase in energetic demand by having access to food ad libitum once they were adults. To limit the possibility for F1 female to compensate, they were starved from day 13 following the blood meal. However, our results (**Fig. 4**) suggests that starvation had no or the opposite effect on longevity: instead of making any cost apparent, we observed a similar decrease in survival in all 4 treatment combinations after the sugar was removed. However, we still detected a global negative effect of maternal exposure and an additional decrease in longevity in exposed mosquitoes from exposed mothers. The presence of cross-generational costs supports the idea that a hormetic response (here the increase of reproductive success) comes at a cost for the organism and is involved in life-history trade-offs (71).

2.5.3 Adult tolerance experiment

When mosquitoes were exposed to a sublethal dose of permethrin at larval stage, they were more likely to survive to a lethal permethrin exposure at adult stage. To our knowledge, this is the first time that such transstadial effect is shown using an entirely sublethal dose of insecticide. It may be

explained by an induction of detoxifying enzymes (123) and/or oxidative-stress (210) related mechanisms. Moreover, it has been recently showed that pyrethroid exposure alters the microbial community of *Anopheles* mosquitoes (211), some of these microbes being able to metabolize pyrethroids (212, 213). While we cannot rule out any of the above mechanisms, they could have acted alone or in concert to detoxify the pyrethroid.

2.5.4 Consequences for resistance evolution

Whether or not permethrin at sublethal concentration contribute to the evolution of resistance mainly depends on the mechanisms involved in the effects observed (mainly fecundity and longevity). Thus, if the observed variation in these traits is 1) inheritable and 2) related to insecticide detoxification or other resistance mechanism, then resistance may evolve. If not, then the effect of permethrin at the tested concentration may be neutral for resistance. While our results – at least for larval development, egg-laying success and fecundity – suggest that the variation indeed has a genetic component, further research should focus on the genetic correlation between the observed effects and insecticide resistance mechanisms.

2.5.5 Consequences for vectorial capacity

We found two major and one minor changes in mosquito traits that may affect its vectorial capacity. The first is the potential increase in egg-laying success, leading to an important increase in the total number of eggs laid. In nature, such increase in reproductive success (with no consequences on egg hatchability) may greatly increase the mosquito to human ratio, which is a critical parameter of vectorial capacity (73). Second, the observed cost in longevity in F1 females may strongly and negatively impact vector competence by reducing the probability that the parasite successfully achieves its development (75). A third any maybe less important point is the highest tolerance of adult mosquitoes to permethrin. The importance of this effect is likely to depend of the vector control tools used, and whether the conferred tolerance provides an advantage for the mosquitoes to bypass the protection conferred by IRS and ITNS. Against resistant mosquitoes ITNs are usually considered more protective than IRS (113, 114, 117).

Finally, which one of these traits has the most important impact on vectorial capacity may depend on the environmental conditions. Indeed, the insecticide may interact with other environmental stressors, such as temperature (94, 214), food availability (95), and the presence of predators (215) or pathogens (216, 217). These additional stressors may constraint the advantages provided by the insecticide to the mosquitoes, and/or add extra energetical costs (e.g. leading to a stronger decrease in longevity across generations). For these reasons, further investigations may benefit from considering ecological conditions to better understand the role of environment in shaping mosquitoes' responses to insecticides.

Chapter 3

Consequences of larval competition and exposure to permethrin for the development of malaria parasites in *Anopheles gambiae*

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3.1 Abstract

Because insecticides are used widely for both agriculture and vector control, they can affect mosquitoes throughout their life cycle. Sublethal exposure to insecticides not only affects the mosquitoes' development and physiology, but may also impact their ability to transmit pathogens, in particular in stressful environments. In this study we assessed how the exposure of larvae or adults to a sublethal dose of permethrin (a pyrethroid) and how larval competition affect the vector competence of *Anopheles gambiae* for the rodent malaria parasite, *Plasmodium berghei*. Vector competence was assessed by measuring prevalence and intensity of infection and the mosquito's longevity. Exposure to the insecticide increased the longevity of mosquitoes after infection, but the effect was only visible in mosquitoes reared in competition. We also found that exposure of larvae or adults to permethrin, but not larval competition, decreased the prevalence of malaria, while larval competition but not exposure increased the intensity of infection. We suggest that exposure to a low dose of pyrethroids might help mosquitoes to deal with a *Plasmodium* infection as a consequence of oxidative stress and antioxidant activity. Our results stress the importance of early-life conditions and insecticide exposure for competence of *Anopheles gambiae* for malaria.

3.2 Introduction

Mosquitoes and other vectors of infectious diseases may often be exposed to sublethal doses of insecticides. On the one hand, when mosquitoes touch the insecticides that are meant to kill them, they can be irritated and fly away before the contact is long enough to kill them. On the other hand, the insecticides that are used in agriculture can runoff and end up at sublethal concentrations in surface or ground water, where they can affect many animals, including aquatic arthropods such as mosquito larvae (218).

Such sublethal concentrations can increase oxidative stress (155, 210), the efficacy of immune responses (Hauser and Koella, submitted) and the activity of detoxifying enzymes (219, 220, 121) and the metabolic cost associated with detoxification. They can also change the behavior of mosquitoes (45, 46, 48, 221), in particular if the insecticide is neurotoxic.

Sublethal concentrations of insecticides also affect the development of malaria and other parasites in mosquitoes. Thus, exposing adult mosquitoes to a low concentration of pyrethroid impedes the development of malaria parasites (128, 222, 223), and the effects of exposing larvae can carry over to adults to influence their vector competence for arboviruses (81, 214) and malaria (95, 224).

Such carry-over effects on ability of mosquitoes to acquire, maintain and transmit its parasite are not limited to insecticides. For example, nutritional stress during the larval stage can impact the susceptibility of *Aedes aegypti* mosquitoes to arboviruses (132, 225, 226), and the vectorial capacity of *Anopheles* mosquitoes for *Plasmodium* (88, 95, 131) or for filarial worms (227).

Since these environmental factors – food limitation for larvae and exposure of insecticides to larvae and adults – may often co-occur in nature, we investigated how their combination would affect the development of malaria in adult mosquitoes, using the mosquito *Anopheles gambiae s.s.,* a pyrethroid insecticide (permethrin), and the rodent malaria parasite *Plasmodium berghei*. In contrast to other studies on the impact of larval exposure, which used high concentrations that kill many larvae or exposed larvae only briefly (e.g. (95, 55, 191, 60, 195)), we used a sublethal concentration throughout the larval period.

3.3 Methods

We used the insecticide-sensitive Kisumu strain of *Anopheles gambiae s.s.* (166) and the ANKA strain of the rodent malaria *Plasmodium berghei* (modified to express green fluorescence protein (GFP)), obtained from the lab of Dr. Heussler at the University of Bern, to assess the consequences of larval competition and of larval and adult exposure to a sublethal concentration of permethrin on the longevity of mosquitoes after infection and on the development of the parasite inside the mosquito. The experiment was run in two blocks with identical methods, and was run in an insectary maintained at 26.5 ± 0.5 °C and 70 ± 5% humidity, with a 12:12 light to dark photoperiod.

3.3.1 Determination of sublethal dose of permethrin

The dose of permethrin was the same as for the experiment described in Chapter 2. Briefly, we tested the effects of 3 concentrations of permethrin (0.1, 0.15 and 0.2 µg/L) on the mortality of individually reared *Anopheles gambiae s.s.* We used a solution of 0.015% volume per volume (118 µg/L) ethanol for the control solution. Freshly hatched larvae were then placed individually in glass petri dishes (4 cm diameter x 1.2 cm height) containing 4 mL of solution, and fed according to the regime described below. Ethanol alone (control solution) was not found to induce significant mortality and, according to the results, we selected the highest concentration of permethrin giving no significant mortality (0.15 µg/L) for the following experiment. Ethanol concentration (for both control and permethrin exposed larvae) was kept at 0.015% v/v (118 µg/L).

3.3.2 Mosquito rearing and permethrin exposure

Freshly hatched larvae (0-3 h old) were reared in glass petri dishes containing 4mL of 0.015% v/v (118 µg/L) ethanol in deionized water with 0.15 µg/L permethrin or no permethrin. The larvae were reared either individually or in groups of 3. They were provided daily with Tetramin Baby® fish food according to their age: 0.04, 0.06, 0.08, 0.16, 0.32 and 0.6 mg/petri dish for ages 0, 1, 2, 3, 4, and 5 or older respectively (196). Note that the this gives strong competition for food in the petri dishes containing three larvae. Upon pupation, mosquitoes were moved individually in 50 mL Falcon™ tubes for emergence. For pupae of the competition treatment, we discarded the petri-dishes in which any larva died, and if more than one female emerged from the same petri-dish, we randomly selected one of them using an random number generator and discarded the other(s) to guarantee independence of data. After emergence, males were discarded, and females were moved to cages and given constant access to a 6 % sucrose solution. 40 hours before infection (so when females were between 1 and 5 days old), half of the females were moved by group of 25 to WHO insecticide-testing tubes and exposed for 2 minutes to permethrin 0.75% treated papers, and the other half was exposed to WHO pyrethroid control papers following WHO exposure protocol (199). Exposed mosquitoes were then moved back to cages according to their treatment. One mosquito died within 24 hours after it was exposed to permethrin (the mosquito was reared individually and not exposed to permethrin at larval stage). No death was recorded after control exposure to mineral oil.

3.3.3 Infection with *Plasmodium berghei*

Two infected mice were available for each block. To control for differences among mice, we split the mosquitoes of each treatment into two cages, one for each mouse. Each mouse was used to feed one cage per treatment (so a total of 8 cages); the order of the treatment was opposite for the two mice. The mice were anesthetized with an intra-peritoneal injection (8.5 mL/kg) of a solution of Xylazine

Xylasol® (20 mg/mL), Ketamine Ketasol (100 mg/mL) and PBS (228), and placed onto each cage for 10 minutes.

Mosquitoes were infected 40 hours after adults were exposed to permethrin or control paper (so 3 to 7 days after emergence). Twenty-four hours before the infection, the temperature was gradually lowered to 19°C and maintained at this level for the rest of the experiment to allow the development of *P. berghei* (229). Mortality was recorded daily, and dead mosquitoes were kept frozen at -20°C.

22 days after infection (when sporozoites are present in salivary glands), mosquitoes were killed by freezing.

3.3.4 Measuring wing lengths

The left wing of dead mosquitoes (including those that died naturally before mosquito were killed) was dissected and the distance from the axillary incision to the tip of the wing was measured (230) with the free software ImageJ (198).

3.3.5 Measuring infection

To determine the infection of the mosquitoes we dissected them in 0.15 M NaCl solution. We counted the oocysts under a fluorescence microscope, and transferred the salivary glands in 1.5 mL Eppendorfs containing 10 µL of Triton X-100 0.05 % and kept at -80°C. The number of sporozoites was later assayed with a real-Time PCR.

DNA extraction

The extraction protocol was modified from Rider et al (231). Salivary glands were first crushed in 125µL of DNAzol® (MRC Inc. Cincinnati, Ohio) using micro pestles. The resulting homogenate was incubated at 55 °C for 20 min, and centrifuged at 20'000 g for 10 min. 100 µL of the supernatant were transferred to a new tube, which had previously been filled with 1.5 µL of Polyacryl carrier (MRC Inc. Cincinnati, Ohio) to increase DNA recovery, and 100 µL of ethanol 100 % were added to the tube to induce DNA precipitation. The tubes were centrifuged at 15'000 g for 8 min, and the supernatant was discarded. The resulting DNA pellet was washed with 600 µL of ethanol 75 % and the tubes were centrifugated at 15'000 g for 5 min. Ethanol was discarded, and DNA pellets were dried using a speedvac at 45 °C for about 20 min. Dry DNA was eluted in 20µL of milli-Q water and kept at -80°C.

Real-Time PCR

Real-Time PCR was performed with a LightCycler 96 ® system (Roche, Switzerland). A master mix was prepared with 4 µL of HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis Biodyne, Estonia), 0.8µL of each of the primers (400 nM final concentration), 3 µL of extracted DNA, and completed with 11.4 µL of Nuclease-Free Water (Qiagen, Germany) to reach a volume of 20 µL. We used primers that amplify a 111 base pairs regions in the block 4 of merozoite surface protein-1 gene (MSP-1) of *P. berghei* ANKA and NK65 strains, as described in (231). Real-Time amplifications steps were set to 95 °C for 15 min

(initial denaturation, required for EvaGreen qPCR Mix), followed by 50 cycles of 3-steps amplification implying i) 95°C for 30sec (denaturation), ii) 53 °C for 45 sec (annealing), and iii) 72 °C for 30 sec (extension). Samples were tested in duplicates in 96 well plates, along with standard dilutions (from 10^8 to 10^1 gene sequences per µL, see below) in duplicates, blanks, and negative control (extracted DNA from uninfected mosquitoes from our lab colony). The use of EvaGreen dye qPCR mix was already tested for *Plasmodium* quantification in (232) and showed satisfying results. Samples were assayed in duplicates and showed a high repeatability (0.976).

MSP-1 Standards

Escherichia coli carrying a plasmid with the 111 base pairs sequence obtained from a PCR amplification were cultured in LB medium. Plasmid DNA was isolated using Wizard® Plus SV Minipreps DNA Purification System (Promega Corp., Wisconsin). DNA concentration in the purified product was measured using Nanodrop (Thermo Fisher Scientific Inc., Massachussetts), and the number of gene sequences was estimated from the known molecular weight of our target sequence. Serial dilutions were finally made to obtain standards of concentrations ranging from 10^8 to 10^1 sequences per µL.

3.3.6 Statistical analyses

All analyses and graphs were done with the software R (version 3.4.4) (200). Significance of the effects were assessed with the Anova function of the *car* library (201), using a type III anova if an interaction was significant, and a type II anova otherwise. Non-significant interactions were dropped from the final models. In cases of significant interactions contrast analyses were done between the factors of interest using *emmeans* (computing Estimated Marginal Means (EMM)) and *pairs* functions of the *emmeans* library in R, with p-values being adjusted using the *mvt* method.

Larval development

Mortality during development was analyzed with Generalized Linear Model (GLM) with a quasibinomial distribution of errors. The response variable was the proportion of dead mosquitoes per petri dish (so, 0 or 1 if larvae were reared individually, and 0, 0.33, 0.67 or 1 if larvae were reared in groups of three. Explanatory variables were competition status and larval exposure to permethrin. Block was included as a co-factor.

We used a Cox's proportional hazard model from the *survival* library in R (204) to analyze the effect of competition and larval exposure to permethrin on development time. Development time was calculated as the number of days from hatching to pupation. For the competition treatment, the average development time for each petri dish was considered as the response variable. Petri dishes in which one or more larva died were censored on the day of the first death recorded. Block was set as a co-factor.

Wing length

We used a Linear Model (LM) to test for any difference of wing length among the larval treatments. The response variable was wing length, and explanatory variables were larval competition status and larval exposure to permethrin. Block was included as a co-factor. As wing length is usually strongly linked to competition, it was not considered as co-factor in other analyses to avoid a false interpretation of the link between competition and the other traits of interest.

Longevity

Post-infection longevity was analyzed with a Cox's proportional hazard model from the *survival* library (204) in R. Day zero was set as the day of infection. 22 days after infection, living mosquitoes were censored. Explanatory variables were larval competition status, larval exposure to permethrin, and adult exposure to permethrin. The blocks and the mice nested within the blocks were included as co-factors. The proportional hazard ratio assumption was tested using the function *cox.zph* from the *survival* library.

Plasmodium infection

To analyze the effect of larval competition and larval and adult exposure to permethrin on *Plasmodium* infection, we first assessed the prevalence of infection in the different treatments and then tested the oocysts or sporozoite load in infected individuals.

A mosquito was considered as infected if we either found 1 or more oocysts on the midgut or if sporozoites were detected after real-time PCR amplification, and it was considered infectious if sporozoites were detected. We used a Generalized Linear Model (GLM) with binomial error distribution to analyze both the prevalence of infection and infectiousness among our different treatments: larval competition, larval exposure to permethrin, and adult exposure to permethrin. The blocks and the mice nested within the blocks were included as co-factors.

Oocysts were counted under a fluorescence microscope. Sporozoite load was quantitatively estimated from real-time PCR amplification results. The effect of the treatments on both oocyst and sporozoite loads were analyzed using a Linear Model (LM). The same variables were included than for the analysis of infection. Response variables were log-transformed to reach normality.

We also tested the proportion of mosquitoes that were infected with oocysts but not sporozoite, which could indicate a delayed parasite development. That proportion was tested using a binomial GLM that included competition, larval exposure and adult exposure as explanatory factors, and the block and the mouse nested within the blocks were included as co-factors. Finally, a regression analysis (LM) tested the association between oocyst load (explanatory variable) and sporozoite load (response variable). Both variables were log-transformed, and the blocks and the mice nested within the blocks were included as co-factors.

3.4 Results

3.4.1 Larval development

Larval mortality ranged from 2.8% to 12.3%. Permethrin exposure ($\chi^{2=}$13.81, df=1, p<0.001) and competition (χ^2=5.68, df=1, p=0.017) significantly increased mortality, and there was a significant interaction between the two factors (χ^2=4.88, df=1, p=0.027. Permethrin increased mortality from 2.8% (95% CI: 1.3 to 6.2%) to 12.3% (8.5 to 17.6 %) (z=3.3, p=0.001) in larvae reared by groups of three, but had a small effect if larvae had been reared individually (from 7.5% (5.2 to 10.7%) to 11.1% (8.3 to 14.7%); contrast analysis: z=1.66, p=0.097).

Almost all larvae (96%) pupated between 7 and 12 days after hatching. The 67 of 1682 larvae that had not pupated by day 12 were removed from the experiment. Competition significantly increased development time from 7.7 ± 0.05 to 9.6 ± 0.07 (mean ± 95% CI) (χ^2=584.9, df=1, p<0.001). There was no main effect of permethrin (χ^2=0.8, df=1, p=0.37), but there was an interaction between the two factors (χ^2=50.74, df=1, p<0.001). Permethrin exposure increased age at pupation in larvae reared individually from 7.41 ± 0.06 to 8.03 ± 0.08 days (mean ± 95%CI) (contrast analysis: z=12.89, p<0.001), but had no effect on larvae reared by groups of three (from 9.55 ± 0.09 to 9.65 ± 0.10 days; z=0.9, p=0.37).

3.4.2 Wing length

Larval competition for food negatively affected wing length ($F_{1,425}$=378.98, p<0.001; with 2.85 ± 0.02 mm for competing larvae against 3.16 ± 0.02 mm for larvae reared individually (mean ± 95%CI)), and larval exposure to permethrin marginally increased wing length from 3.01 ± 0.03 mm to 3.03 ± 0.02 mm ($F_{1,425}$=4.99, p=0.026). However, there was a significant interaction between the two factors ($F_{1,425}$=4.90, p=0.027); permethrin exposed mosquitoes were slightly larger than unexposed ones if larvae had been reared in groups of three (from 2.84 ± 0.02 mm to 2.88 ± 0.03 mm; contrast analysis: t=-2.23, df=420, p=0.026), but exposure had no effect if larvae had been reared individually (3.15 ± 0.02 mm (exposed), against 3.17 ± 0.02 mm (unexposed); contrast analysis: t=0.85, df=420, p=0.39).

3.4.3 Post-infection longevity

Survival 22 days after the blood meal ranged from 52.3% (95 %CI: 37.9 to 66.2%) for mosquitoes reared in competition and exposed to permethrin only as adults to 81.6% (66.6 to 90.8%) for mosquitoes reared in competition and exposed both as larvae and adults. Competition slightly decreased longevity (χ^2=3.70, df=1, p=0.054). Neither larval (χ^2=1.58, df=1, p=0.21) nor adult (X^2=0.36, df=1, p=0.55) exposure to permethrin had a main effect on longevity, but there was an interaction between competition and the two exposure (X^2=4.33, df=1, p=0.037). Contrast analysis showed that when mosquitoes were reared individually, there was no difference in post-infection longevity between the

treatments (all p>0.68, **Fig. 1a**). However, in mosquitoes reared in groups of three, those that were exposed to permethrin as larvae and as adults lived longer than those that were only exposed as adults (z=2.8, p=0.025), or those that were not exposed to permethrin at all (z=2.48, p=0.055).

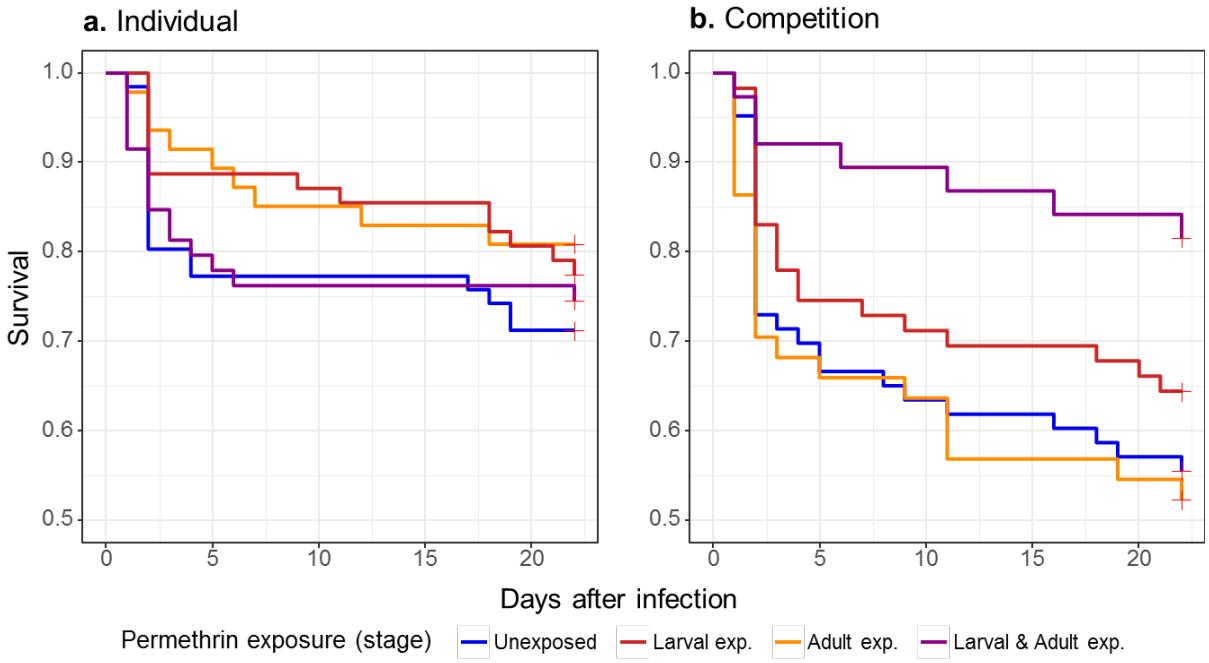

Figure 1. Left panel (a) shows post-infection longevity according to permethrin exposure for mosquitoes that had been reared individually. N=234. Right panel (b) shows the same treatments but for mosquitoes that had been reared in competition. N=205. Scale of the y axis was fixed between 0.5 and 1 to improve visibility.

3.4.4 *Plasmodium* infection

The output of the models for malaria infection (infection prevalence, infectiousness, sporozoite load and oocyst load) are presented in **Table 1.** Reported results were consistent among the two experimental blocks.

Overall infection

303 mosquitoes were killed and dissected 22 days after infection. Oocysts could be counted in all of these, while salivary glands were recovered (and sporozoite load determined) in 280. To analyse the overall prevalence of infection (that is, independent of the parasite's stage), we used only mosquitoes for which both oocyst and sporozoite load could be determined. 75.4 % (95% CI: 69.9 to 80.0 %) of the mosquitoes were infected. Competition status did not affect the proportion of infected individuals, with 76.7% (69.6 to 82.5%) in mosquitoes reared individually, against 73.5% (58.8 to 80.7%) in those reared by groups of three (**Table 1**). In unexposed mosquitoes, the prevalence was 89.3% (80.3 to 94.5%). Exposure as larvae (67.5% (56.5 to 76.9%), as adults (69.1% (56.0 to 79.7%) and as both (74.0% (62.9 to 82.7%) reduced the prevalence in similar ways (**Table1, Fig. 2a**).

Table 1. Results of the models for the overall infection prevalence (proportion of mosquitoes infected with either sporozoites, oocysts, or both), infectiousness (proportion of mosquitoes infected with sporozoites), sporozoite load, and oocyst load. The Chi-square values, the degrees of freedom and the p-values are given. Significance ($p<0.05$) is indicated in bold and with an asterisk, trends (p between 0.05 and 0.1) is indicated in bold with a "+" symbol.

	infection prevalence		***infectiousness***		***sporozoite load***		***oocyst load***	
	χ^2_{df}	p	χ^2_{df}	p	χ^2_{df}	p	χ^2_{df}	p
competition	0.43_1	0.51	0.13_1	0.71	3.79_1	0.053^+	12.64_1	**<0.001***
larval exposure	10.14_1	**0.001***	11.94_1	**<0.001***	0.07_1	0.78	4.53_1	**0.035***
adult exposure	8.24_1	**0.004***	5.64_1	**0.018**	1.68_1	0.20	4.22_1	**0.042***
competition: larval exposure	0.06_1	0.80	0.01_1	0.91	0.06_1	0.81	8.21_1	**0.005***
competition:adult exposure	0.17_1	0.68	0.01_1	0.94	0.38_1	0.54	1.05_1	0.31
larval exposure: adult exposure	7.87_1	**0.005***	6.38_1	**0.011***	0.18_1	0.67	0.46_1	0.50
competition:larval exposure: adult exposure	0.01_1	0.93	0.15_1	0.70	1.23_1	0.27	1.68_1	0.20

Sporozoites

Salivary glands were recovered in 280 individuals, among which 183 were found to be infected with sporozoites (65.4 % (95% CI: 59.6 to 70.7 %)). In unexposed mosquitoes, the prevalence was 81.3% (71.1 to 88.5%). Exposure as larvae (54.5% (43.5 to 65.2%), as adults (61.8% (48.6 to 73.5%) and as both (63.5% (53.5 to 73.2%) reduced the prevalence in similar ways (**Table1, Fig. 2b**). Competition was not found to affect mosquitoes' infectiousness (64.4% (56.8 to 71.4%) for individually reared larvae, against 66.7% (57.7 to 74.6%) in mosquitoes reared in groups of three (**Table1**).

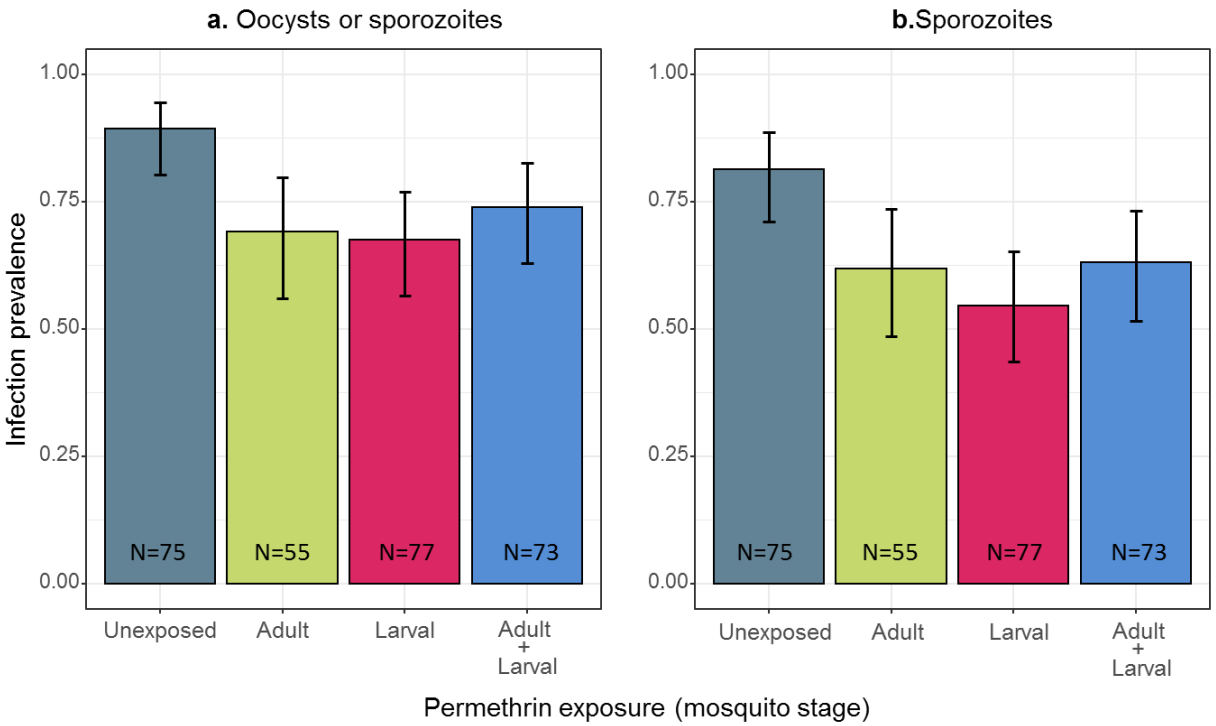

Figure 2. Left panel (a) shows the prevalence of infection of the mosquitoes 22 days after the infectious bloos meal, regardless of the parasite stage (oocyst or sporozoites). Colors indicate mosquitoes' exposure to permethrin. Right panel (b) shows mosquitoes' infectiousness, i.e. the proportion of mosquitoes harboring sporozoites 22 days after the infectious blood meal. Error bars show the 95% confidence intervals.

The sporozoite load of sporozoite-positive mosquitoes ranged from 10 to 68'570 (mean 4725 ± 95% CI 1549). Mosquitoes reared in groups of three tended to have fewer sporozoites (2623.05 ± 1135.78) than individually reared mosquitoes (6286.38 ± 2543.25) (**Table 1, Fig. 3**). Permethrin exposure did not affect sporozoite load (**Table 1**).

Oocysts

On the 303 individuals dissected, 163 individuals harbored oocysts (53.8 % (95% CI: 48.2 to 59.3 %)). The number of oocysts varied from 1 to 110 (mean ± 95% CI: 17.1 ± 2.62). Mosquitoes reared in groups of three had fewer oocysts (8.4 ± 2.8) than mosquitoes reared individually (15.1 ± 3.7; **Table 1**). Mosquitoes exposed as adults tended to have fewer oocysts (10.5 ± 3.9) than unexposed adults (14.5 ± 3.5; **Table 1**). There was also a lower oocyst load in mosquitoes exposed as larvae (12.0 ± 3.5 against 14.0 ± 4.0; **Table 1**), but this effect depended on whether larvae had reared individually of in groups of three. Permethrin at larval stage non-significantly decreased oocyst load of mosquitoes reared individually (contrast analysis; t=1.9, df= 155, p=0.057), and increased oocyst load of those reared in groups of three (t=-2.1, df=155, p=0.03).

28 out of 280 mosquitoes harbored oocysts but no sporozoites (10.0 % (95% CI: 7.0 to 14.1%)). This proportion was not affected by any of the tested treatments (p>0.14) and no interaction was significant

(p>0.76). The number of oocysts was also found to be positively correlated with sporozoite load ($F_{1,119}$=56.28, p<0.001).

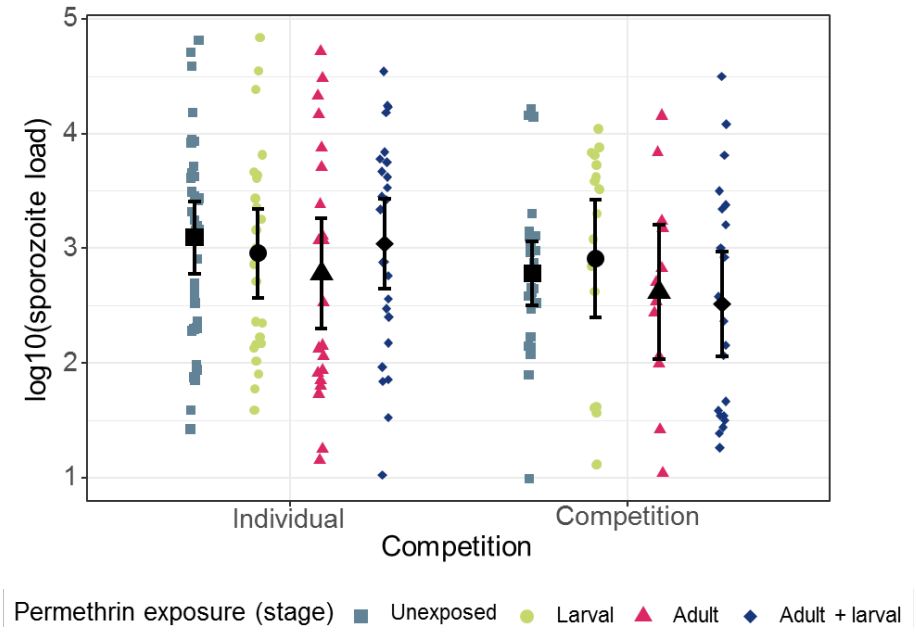

Figure 3. Sporozoite load in mosquito salivary glands. Only mosquitoes harboring sporozoites are shown. All treatments are represented (see figure legend). Error bars show the 95% confidence intervals. N=183.

3.5 Discussion

In our study, we assessed the consequences of intraspecific larval competition, larval exposure and adult exposure to permethrin on post-infection longevity and on the development of *P. berghei* inside *An. gambiae* mosquitoes. We found that permethrin reduced the prevalence of infection and, to some extent, increased its post-infection longevity.

3.5.1 Development and adult size

Permethrin significantly delayed pupation in individually reared larvae, and showed a similar trend in larvae reared in competition. Thus the insecticide affected the ability of mosquitoes to invest in development, either directly through neurotoxic effects or indirectly via the costs associated to detoxification, metabolization or sequestration of permethrin (101, 102, 127). That a trade-off exists between detoxification and development would imply that the cost may be more apparent in the case of food restriction. Our results are consistent with this idea, as permethrin slightly increased mortality of larvae reared in competition by 9.5%, whereas the increase of mortality in individually reared larvae (by 3.6%) was not significant.

Intraspecific competition led mosquitoes to become smaller adults (wing length reduced by 9.5%), while the effect of permethrin depended on competition. Mosquitoes reared in competition and exposed to permethrin were marginally larger (+2.1%) than their unexposed counterparts, while no

difference was found in individually reared mosquitoes. Because mortality was also higher in the former treatment, this may indicate that permethrin primarily kills the smaller and weaker individuals, selecting for larger ones. In individually reared mosquitoes, the absence of a negative effect of permethrin on adult size, despite the presumed costs of being exposed, could be explained by their ability to catch-up at the end of their development. This phenomenon called *compensatory growth* is often observed when the environment is changing between early and late development phases (233). In our experiment the environment remained stable, but it is likely that the perception of it by the larvae did change: a fixed concentration of insecticide is unlikely to represent the same toxicity for stage I or stage IV larvae, for example. Therefore, later stages, by being less affected by the insecticide, might have experienced compensatory growth and reached a normal adult size, as it has been observed in different settings (234, 235).

3.5.2 Longevity post-infection

Competition decreased post-infection longevity by about 20% in every treatment but one. This effect of competition is in line with previous work (88) and may be a consequence of the reduced resource availability in small females (236), which likely limit their immune responses (e.g. (84)). In addition, while we found no effect of permethrin exposure on mosquitoes reared individually, those reared in competition had an increased longevity when they were exposed both as larvae and as adults. These results do not support previous studies that found no or negative effect of insecticide on post-infection survival following larvicidal (*Bti*) (95) or pyrethroid exposure (128, 237). Indeed, our results suggest that the insecticide may positively interact with mosquito immunity (as suggested by the results on prevalence) and alleviate the costs of the infection. In individually reared mosquitoes, however, it might be that the high survival rate 22 days after the infection (ca. 80%) have hidden any beneficial effect of permethrin exposure.

3.5.3 Parasite prevalence

In contrast to what was found for survival and in other studies (88, 95), intraspecific larval competition did not influence the prevalence of infection or the proportion of infectious mosquitoes. Because of the limited energetic resources that could be invested in immunity, a higher prevalence of infection in mosquitoes reared in competition was expected. Possibly, mosquitoes from competition treatment suffered from an initially higher prevalence than individually reared mosquitoes, but because this is likely to have lead to the death of the most infected mosquitoes, final infection prevalence may have decreased to reach similar values than that of individually reared mosquitoes.

In addition, we found that permethrin exposure at both larval or adult stage significantly reduced both the prevalence of infection and the mosquitoes' infectiousness, although the effects were not additive. These results are in accordance with several studies that found reduction of *Plasmodium* infection

prevalence after adult mosquitoes were exposed to deltamethrin (pyrethroids) (128, 223, 237), bendiocarb (carbamate) or DDT (organochlorine) (119) (but see (238)). Our findings corroborate and complements these results by showing that exposure at both larval and adult stages – even at very low dose– have similar effects on infection prevalence.

While pyrethroid insecticides directly affect the development of *Plasmodium* (222, 223), it is unlikely to be the only mechanism involved, as larval and adult exposure led to a similar decrease in prevalence. Alternatively, the observed effects may be due to an indirect effect of the insecticide on mosquito physiology and immunity, as already suggested but for different insecticides (119). They are several non-mutually exclusive ways through which insecticides can affect insect immunity (reviewed in (57)). Those includes effects on the number of hemocytes (239), the activity of immune enzymes (240, 241), or oxidative stress. Indeed, pyrethroid insecticides are a major source of reactive oxygen species (ROS) in exposed organisms (155, 210, 242), and it is well known that ROS are a common immune defense against a large variety of pathogens (153), including *Plasmodium* (150–152). However, oxidative stress per se may also cause various damages to an organism (243). After exposure to a pyrethroid, the intrinsic toxicity of ROS is considered as deleterious as the neurotoxic effect of the insecticide itself (147). It seems therefore unlikely for oxidative stress itself to be the mechanism helping exposed mosquitoes to survive a malaria infection. Possibly, the antioxidant and not the oxidant activity helps exposed mosquitoes to better deal with a *Plasmodium* infection. Indeed, antioxidants are very important to protect organisms from cell damages and lipid peroxidation in the case of an insecticide exposure (220), which explains why elevated antioxidant activity was often found to be associated pyrethroid tolerance (147, 210, 244). Similarly, beside the increased oxidative stress in the midgut following and infectious blood meal, a systemic increased expression of antioxidant is triggered to prevent oxidative damages in the mosquitoes (150, 245). In line with this idea, two important cytosolic antioxidants (Cu-Zn SOD2 and SOD3A) have been found to be overexpressed both after an infectious blood meal (245, 246) and in pyrethroid tolerant *An. gambiae* (210), and are therefore involved in the physiological response of both stressors. This altogether supports the idea of an antioxidant-based mechanism used in both insecticide and *Plasmodium* responses. This may also work the other way around : in a recently published study, *Anopheles* mosquitoes previously infected with *P. berghei* were found to be less affected by permethrin (they showed a shorter period of feeding inhibition) than uninfected mosquitoes (48). This study together with the present results show with concrete examples that both vector competence for *Plasmodium* and mosquito response to permethrin can be significantly modified in presence of the other stressor.

3.5.4 Parasite load

In contrast to what was found with infection prevalence, both oocyst and sporozoite load were negatively affected by larval competition. While there was a marginal effect of permethrin exposure on oocyst load, it was not the case for sporozoites, which are the infectious stage. The lower oocyst and sporozoite loads in mosquitoes reared in competition may possibly be explained by the difference in body size. In larger hosts, malaria parasites might have had more energetic resources to develop. It should however be noted that this result does not support the findings of Emami and her team who did not notice any correlation between *P. falciparum* load and *A. gambiae* body size (247). Alternatively, it can also be explained by the higher mortality observed in mosquitoes from competition treatment: heavily infected may not have survived and led to the observed difference in sporozoite load. That permethrin exposure did not impact parasite load whereas it does for infection prevalence is more surprising and differs from previous study using deltamethrin insecticide (128). One possible explanation is that the high variability observed in sporozoite load, by decreasing statistical power, affected our chance to detect significant differences between our treatments (**Figure 3**).

To conclude, our results show that sublethal exposure to permethrin at larval or adult stages can affect the way mosquitoes respond to a *Plasmodium* infection. We found that exposure to permethrin increased the post-infection longevity of mosquitoes reared in competition, and exposure at both larval and adult stages reduced infection prevalence. To explain these results, we propose oxidative stress and antioxidant activity to be plastically modulated by the insecticides, which in turns helps mosquitoes to limit parasite development. In addition, we showed that larval competition for food – ubiquitous in nature – has direct effects on the outcome of an infection in terms of survival and parasite load, and could modify the way insecticides affect mosquitoes development and survival. Altogether, this suggests that both early life conditions and adult exposure can strongly affect mosquito vector competence.

Chapter 4

Larval exposure to an insecticide and competition for food modulate the immune responses of adult *Anopheles gambiae*

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4.1 Abstract

Because insecticides are extensively used for agriculture and vector control, mosquito larvae may be exposed to low concentrations of insecticides in their aquatic environment. These toxic compounds not only likely affect the development of the larvae, but may also have long-lasting effects on the adults, potentially affecting their vectorial capacity. Such an impact may be expected to be more severe when mosquitoes are undernourished. In the present study, we investigated with the mosquito *Anopheles gambiae* whether exposing larvae to a sub-lethal dose of permethrin (a pyrethroid) and forcing them to compete for food affect the immune response of the adults. We found for individually reared larvae that a low dose of permethrin increased the degree to which a negatively charged Sephadex bead was melanized and slowed the replication of injected *Escherichia coli*. However, if mosquitoes had been reared in groups of three (and thus had been forced to compete for food) permethrin had lower impact on the efficacy of the immune response. Our results show how larval stressors can affect the immune response of adults, and that the outcome of exposure to insecticides strongly depends on environmental conditions.

4.2 Introduction

The immune system of mosquito vectors underlies their susceptibility to parasites, and thus their ability to transmit pathogens to humans (248, 249). The strength of their immune response is strongly influenced by their genetic background (250). Characterizing genes that influence the immune response is becoming routine and new tools are making it easier to transform mosquitoes with such genes, raising hopes that generating a parasite-resistant mosquito population may soon be feasible (251). But we should not forget that the immune response and vectorial competence are also influenced by the mosquitoes' ecology. Vectorial competence is, for example, strongly influenced by the bacterial microbiota the mosquitoes acquire as larvae (252), and the susceptibility of *Aedes* mosquitoes to arboviruses and the response of *Anopheles* mosquitoes to malaria parasites depend on the temperature of the larval environment and food availability to the larvae (131, 253, 254). Similarly, the strength of the immune response of adult *Anopheles* mosquitoes is influenced by the amount of food available to the larvae (255).

An aspect of the environment that is becoming increasingly important is the presence of insecticides. Indeed, because insecticides are used extensively in agriculture and vector control, they are often found in water of agricultural areas (256), where mosquito larvae are exposed to them. Although their concentration is often so low that they do not kill all the larvae, they have an impact on the mosquitoes' life- history traits (reproductive success, adult longevity, sex ratio (55, 94, 191, 192)). In particular, their effects can carry over to adults to influence their vector competence for arboviruses (81, 214) and malaria (95, 224). Such effects may lead to a situation where the insecticides used to kill mosquitoes may make them more sensitive to infection by the parasite we would like to control.

The effect of sub-lethal doses of insecticides on vectorial competence is likely to be linked to their impact on the immune response. Understanding the interaction between insecticides and the immune response of mosquitoes is therefore crucial to better predict the impact of insecticides on control.

Exposure to insecticides can affect the immune response of insects in several ways (57). Thus, botanical insecticides (257, 258), an insect growth regulator (259) and a pyrethroid (241) decrease phenoloxidase activity, which is involved in the melanization immune response, and botanical insecticides (133), organophosphates and organochlorines (134) impact the number of hemocytes, which determines the strength of phagocytosis.

Although these results show that a weak exposure to insecticides can perturb the immune system of insects, it is not known whether exposure of larvae will carry over to affect the immune response of adults. The goal of our project was to test whether such a carry-over exists. We therefore investigated the effect of exposing mosquito larvae to the pyrethroid permethrin, an insecticide widely used for agriculture and vector control, on two immune responses of adults. (i) The melanization capacity of mosquitoes is an important component of the immune response of insects, and helps to clear many

pathogens (135, 139) including malaria parasites (140, 141). The melanization response is a single component of the insect's immune response. (ii) In contrast, the ability of the insect to suppress the growth of bacteria can be a consequence of several branches of the immune response and involves antibacterial compounds such as defensin, the use of reactive oxygen species, phagocytosis and the melanization response (135).

In addition to exposing mosquitoes to the insecticide, we included larval competition in our design as a mechanism through which the food available to larvae may be limited. Since larval food affects many aspects of the mosquito's life-history (87), including vectorial competence (131, 254) and immune responses (255), we expected that an effect of the insecticide would be more likely to be seen when larvae are forced to compete for food. Also, larval competition and an exposure to an insecticide may have interacting effect on larval development and adult wing size (260), which in turn affects mosquito immunocompetence (255, 261). We thus additionally recorded development time, survival and adult size to assess for any effect of our larval treatments.

4.3 Material and Methods

We used the insecticide-sensitive Kisumu strain of *Anopheles gambiae s.s.* (166) to investigate the melanization and the antibacterial responses of the mosquito in two separate experiments. In a first experiment we tested the effects of larval competition for food and permethrin exposure on melanization capacity by injecting Sephadex™ beads inside the thorax of mosquitoes and measuring the amount of melanin that was deposited. This experiment was repeated in two separated blocks. In the second experiment, the same stressors were tested for their effects on the mosquito's response against bacterial infection. We injected *E.coli* bacteria into the mosquitoes' thorax, and assayed the development of the bacteria by counting them one day later. This experiment was run in one block. Both experiments used the same protocol to rear larvae and the same concentration of permethrin to expose larvae (as described below).

All experiments were run in an insectary maintained at 26.5 ± 0.5 °C, 70 ± 5% humidity and a 12:12 light to dark photoperiod.

4.3.1 Determination of sublethal dose of permethrin

In a preliminary experiment we determined a concentration of permethrin that (in our laboratory conditions) was just below a threshold that killed the mosquito larvae before they were able to emerge as adults. Five different concentrations of permethrin were tested: 0.04, 0.1, 0.15, 0.3 and 0.8 µg/L. Permethrin solutions were made from a 1 µg/mL stock solution of solid permethrin (Sigma-Aldrich Inc., St. Louis, Missouri) dissolved in pure ethanol. Between 40 and 50 larvae per concentration were reared individually in glass petri dishes (4 cm diameter x 1.2 cm height) containing 4 mL of 0.004 % to 0.08 % (volume per volume) ethanol containing the desired concentration of permethrin. For the control, a

solution of 0.08 % v/v (631 µg/L) ethanol was used. This latter concentration was not found to induce significant mortality (1/48 (2.1% (95% CI: 0.4 to 10.9%)) tested against a mortality of 0/48 using a 2-sample test for equality of proportions (*prop.test* in R)). Based on the results, we used in the main experiments a permethrin concentration of 0.1 µg/L, which did not cause significant mortality compared to controls. The concentration of ethanol used as control was fixed at the corresponding concentration of 0.01 % v/v (78.9 µg/L).

4.3.2 Mosquito rearing and permethrin exposure

Freshly hatched *An. gambiae* larvae (0-3h old) were put into glass petri dishes (4 cm diameter) containing 4mL of 0.01% ethanol supplemented with 0.1 µg/L or no permethrin. Larvae were reared either individually or, to induce among-larvae competition, in groups of three. Tetramin Baby® fish food was provided daily according to the age of the larvae: 0.04, 0.06, 0.08, 0.16, 0.32 and 0.6 mg/larva at age 0, 1, 2, 3, 4, and 5 or more days, respectively (196). Each petri dish was provided with an amount of food calculated for 1 larva, which implies that, on average, larvae from the competition-treatment received about one third of the food received by individually reared larvae. Pupae were transferred individually to 50mL Falcon™ tubes. If more than one female emerged from a petri dish where a group of three had been competing, we selected one randomly for further analysis to guarantee independence of data. Males and unselected females were discarded. The selected females were transferred to 21 x 21 x 21 cm cages according to their treatment and age (one cage per day of emergence and treatment), where they had constant access to a 6% sucrose solution.

4.3.3 Bead injection

Melanization ability was tested by inoculating 4-day-old adult females with negatively charged carboxymethyl Sephadex® C-25 beads (Sigma-Aldrich Inc., St. Louis, Missouri) according to (262). We anesthetized female mosquitoes in a Falcon™ tube placed on crushed ice for 5 to 10 minutes, and then injected with a glass microcapillary one bead (50-130 µm diameter) into the thorax of the mosquito. Injected females were transferred to cages, and were killed by freezing 24 hours after injection. Mosquitoes were dissected in 0.1% methyl green colored solution to facilitate bead recovery, and pictures of recovered beads were taken with a microscope with 20x magnification.

4.3.4 Measurement of melanization response

We assessed the melanization response in two ways. We found a qualitative measure of melanization by determining visually whether a bead was unmelanized or was melanized to some degree. We found a quantitative measure of melanization by estimating the amount of melanin deposited on a bead. To do so, we used the software ImageJ v1.51 (198). For each image, the color spectrum of unmelanized parts of the bead was identified and filtered from the bead, so that most of the color on the beads was

due to melanin. We then measured the *mean gray value* of each (filtered) bead, giving 0 for entirely white beads, and 256 entirely black beads. We also estimated the size of the bead by measuring with ImageJ its diameter.

4.3.5 Measurement of anti-bacterial response

We assessed the efficacy of mosquito's antimicrobial response by measuring bacterial growth within the mosquito. We used ampicillin-resistant *E.coli* (*dh5 alpha* strain). Four days after emergence, mosquitoes were anesthetized on ice for 2-5 min and inoculated with 3'500 *E.*coli (0.2 µL of bacteria solution) in the thorax using glass microcapillaries. We kept the inoculated mosquitoes for 24 hours in 21 x 21 x 21 cm plastic cages and then assayed the proportion that survived and measured the bacterial load in the surviving mosquitoes. To do so, mosquitoes were briefly anesthetized on ice, transferred in Eppendorf tubes and crushed using micro-pestles in 200µL of Luria-Bertani broth containing 150µg/mL ampicillin (LA). The homogenate was diluted 20-fold in LA, and 100 µL of this dilution were spread on LA agar plates. The agar plates were incubated at 37°C overnight, and bacteria colonies were counted. The number of *E.coli* colonies was used as a measure of bacterial load in the mosquitoes.

Injection doses were prepared by measuring the absorbance at a wave-length of 600 nm of ampicillin resistant *E.coli* that had been grown overnight in LA at 37°C, and comparing this absorbance with a standard curve that had been made prior to the experiment using *E.coli* solutions of known concentration. Serial dilutions were made until the desired absorbance was reached, which corresponded to 17.5 x 10^6 *E.coli* per milliliter (3'500 bacteria per injection). The solution was kept on ice during the manipulation to avoid further bacterial growth, and a fresh solution was prepared every day of injection.

4.3.6 Statistical analyzes

All analyses and graphs were done with the software R (version 3.6) (200). Significance of the tested variables were assessed with the Anova function of the *car* library (201), using a type III anova if an interaction was significant, and a type II anova otherwise. For LMMs, normality of residuals was visually checked and homoscedasticity was tested using a Breusch-Pagan test (*bptest* function of the *lmtest* library (263) in R). In cases of significant interactions, contrast analyses were done between the factors of interest using *emmeans* (computing Estimated Marginal Means (EMM)) and *pairs* functions of the *emmeans* library in R, with p-values being adjusted using the *mvt* method.

Larval development

Larval mortality was analyzed with a Generalized Linear Model (GLM) with quasibinomial errors, where the mortality in each petri dish was set as the response variable (mortality being 1 or 0 for individually reared larvae, and 1, 0.67, 0.33 or 0 for larvae reared by groups of three). Explanatory variables were competition (individually reared vs reared in groups of three) and larval exposure to permethrin

(exposed vs unexposed). The two experiments (three blocks in total) were analyzed together, and block (A, B or C) was also included in the model to test for differences in mortality.

Larval development time is the number of days from hatching to pupation. We used Cox's proportional hazard mixed-effect models from the *coxme* library in R (264) to analyze the effect of competition and larval exposure to permethrin on development time. For the competition treatment, the average development time for each petri dish was considered as the response variable. Petri dishes in which one or more larva died were censored on the day the first larva died. Block was set as a random factor.

Wing size

We used a Linear Mixed-effect Model (LMM) to test for the effects of competition and larval exposure to permethrin on wing length. Wings from dead mosquitoes were dissected and the distance from the axillary incision to the tip of the wing was measured and analyzed (230). As noted above, we only analyzed females, and at most one individual per petri dish. Block was set as a random factor.

Melanization

We analyzed the qualitative measure of the melanization response, so the proportion of melanized beads, with a GLMM with binomial distribution of errors. We analyzed quantitative melanization, so the grey value of the beads, with an LMM that assumes a normal distribution of errors. To reach normality of residuals, we used the square-root of the difference between the highest grey-value observed (233.4) and the grey value of the bead. For both analyzes, we used competition (individually reared vs reared in groups of three) and larval exposure to permethrin (exposed vs unexposed) as explanatory factors, bead size as a covariate and block as a random factor

Antibacterial response

The effect of competition or larval exposure to permethrin on the number of bacteria found in mosquitoes 24h after the injection was tested using an LMM. The response variable was log-transformed to reach normality of residuals. We used competition (individually reared vs reared in groups of three) and larval exposure to permethrin (exposed vs unexposed) as explanatory factors. Furthermore, since the bacterial stock solution was made fresh every day, we set the day of injection a random factor.

4.4 Results

We reared 1343 larvae individually and 2520 in competition.

4.4.1 Life-history

Mortality ranged from 2.9% to 13.4%. Permethrin increased mortality from 2.9% (95% CI: 1.9 to 4.5%) to 8.1% (6.3 to 10.3%) in individually reared larvae and from 3.9% (2.4 to 6.2%) to 13.4% (10.5 to 16.9%) in larvae reared in competition. While the effects of permethrin (χ^2=49.07, df=1, p<0.001) and

intraspecific competition (χ^2=9.36, df=1, p=0.002) were significant, their interaction was not (χ^2=0.52, df=1, p=0.472). There was no significant difference among blocks (χ^2=5.26, df=1, p=0.072).

Development time from hatching to pupation ranged from 7 to 13 days. Larval competition increased development time from 7.95 ± 0.04 (mean ± 95% CI) to 9.13 ± 0.06 days (χ^2=321.55, df=1, p<0.001). Permethrin increased the development time of individually reared larvae from 7.80 ± 0.04 days to 8.10 ± 0.05; contrast analysis: z=-9.57, p<0.001), but did not affect the development of larvae reared in competition (9.11 ± 0.10 days vs. 9.15 ± 0.08, contrast analysis: z=0.425, p=0.67). Thus, the main effect of exposure to permethrin was not significant (χ^2=0.18, df=1, p=0.671), but its interaction with competition was (χ^2=37.41, df=1, p<0.001).

4.4.2 Wing length

Whereas competition reduced wing length from 3.18 ± 0.02 mm (mean ± 95% CI) to 2.83 ± 0.02 mm (χ^2= 950.02, df=1, p<0.001), larval exposure to permethrin slightly increased wing length from 2.98 ± 0.03 to 3.01 ± 0.03 mm (χ^2= 5.62, df=1, p=0.018). There was no interaction between larval exposure and competition (χ^2= 0.27, df=1, p=0.604).

4.4.3 Melanization

We inoculated 582 mosquitoes, of which 42 died in the first 24 hours and were therefore not included in the melanization assay. The mortality of mosquitoes reared in competition (9.3% (95% CI: 6.7 to 12.9%)) was about twice that of individually reared mosquitoes (4.7% (95% CI: 2.8 to 7.9%)) (χ^2=5.18, df=1, p=0.023). Mortality was affected neither by larval exposure to permethrin (χ^2=0.86, df=1, p=0.353) nor by the interaction between the two factors (χ^2=0.07, df=1, p=0.796).

The injected bead was recovered in 457 of the remaining 540 mosquitoes; 399 of these were at least partially covered by melanin. Mosquitoes reared in competition were less likely to melanize their bead (82.4% (95% CI: 77.1 to 86.7%)) than those reared individually (92.7% (88.4 to 95.4%) (χ^2=4.33, df=1, p=0.037, **Fig. 1**)), mosquitoes exposed to permethrin as larvae were more likely to melanize their bead (91.2% (86.8 to 94.2%)) than unexposed ones (83.4% (78.0 to 87.7%)) (χ^2=5.38, df=1, p=0.020, **Fig. 1**)). The interaction between exposure and competition was not significant (χ^2=1.17, df=1, p=0.279).

In mosquitoes that had melanized their bead at least partially, the amount of melanin – estimated by the mean grey value of the bead – also differed according to the treatment. Beads from mosquitoes reared in competition had less melanin (grey value of 114.1 ± 9.0 (mean ± 95 % CI)) than those from individually reared mosquitoes (153.8 ± 8.9) (χ^2=4.11, df=1, p=0.043), whereas larval exposure had no global effect on the degree of melanization (χ^2=1.74, df=1, p=0.188). However, there was an interaction between competition and larval exposure (χ^2=5.50, df=1, p=0.019, **Fig. 2**). Contrast analysis showed that in individually reared mosquitoes, permethrin increased the amount of melanin deposited on beads (from 146.9 ± 13.6 to 160.0 ± 11.9) (t=2.00, df=393, p=0.046, **Fig. 2**), whereas there was no effect

of the insecticide in the competition treatment (119.8 ± 12.7 for unexposed vs. 108.7 ± 12.9 for exposed mosquitoes) (t=-1.32, df=393, p=0.188), **Fig. 2**).

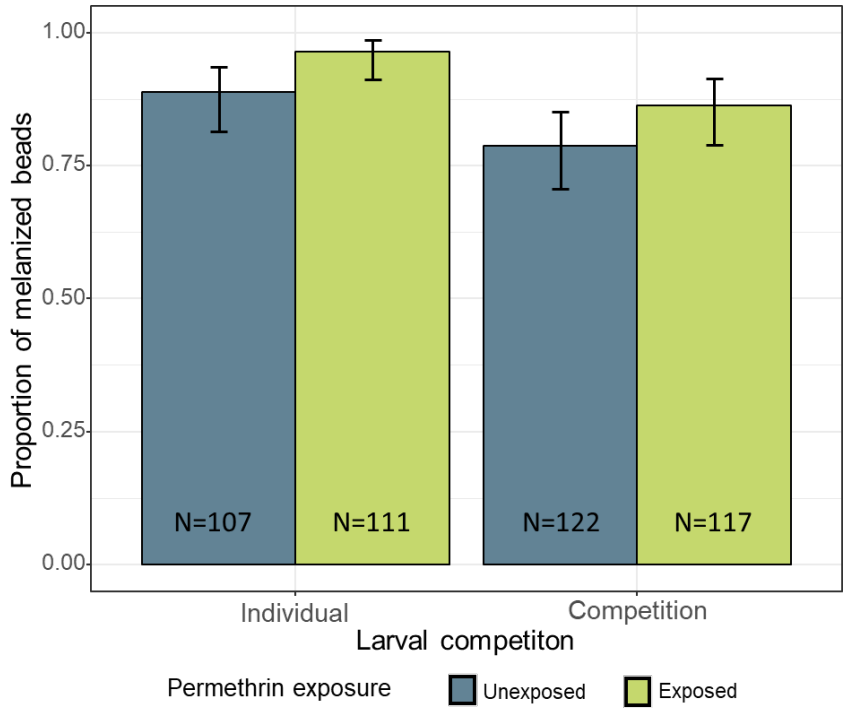

Figure 1. The proportion of beads that were at least partially covered by melanin according to larval competition and exposure to permethrin. Error bars show the 95% confidence intervals.

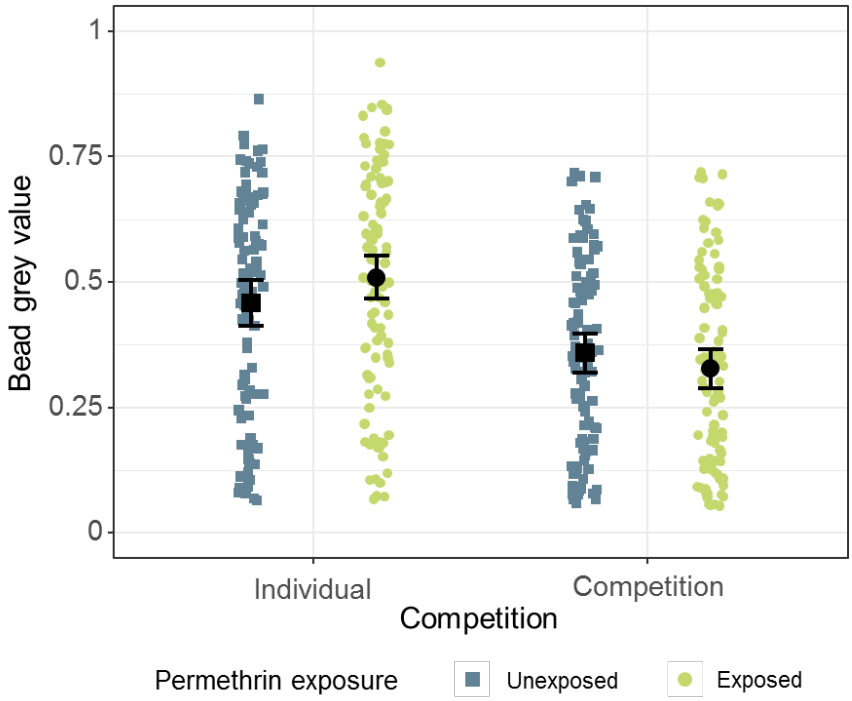

Figure 2. Amount of melanin deposited on beads (fully unmelanized beads are excluded). To help visualization, the grey value was rescaled so 0 means that the bead is white, while a grey value of 1 means that the bead is entirely black. N=399. Error bars show the 95% confidence intervals of the means.

4.4.4 Antibacterial response

We inoculated 195 mosquitoes with *E.coli*, of which 8 died within 24 hours. The mortality was independent of larval competition (χ^2=0.60, df=1, p=0.44) and of exposure to permethrin (χ^2=0.00, df=1, p=0.98).

The bacterial load 24 hours after inoculation was higher in the competition treatment (4797.5 ± 963.7 (mean ± 95% CI)) than for the individually reared mosquitoes (3379.1 ± 831.6) (χ^2= 15.95, df=1, p<0.001), but there was no main effect of larval exposure to permethrin (χ^2=1.70, df=1, p=0.193). However, there was a significant interaction between the two factors (χ^2=7.83, df=1, p=0.005): in individually reared mosquitoes, larval exposure decreased the number of bacteria (from 3941.3 ± 1227.1 in unexposed to 2840.8 ± 1150.0 in exposed mosquitoes) (t=-2.667, df=180, p=0.008, **Fig. 3**), while it had no effect on mosquitoes reared in competition (4243.3 ± 1053.5 in unexposed vs. 5363.4 ± 1651.4 in exposed mosquitoes) (t=1.284, df=183, p=0.201, **Fig. 3**).

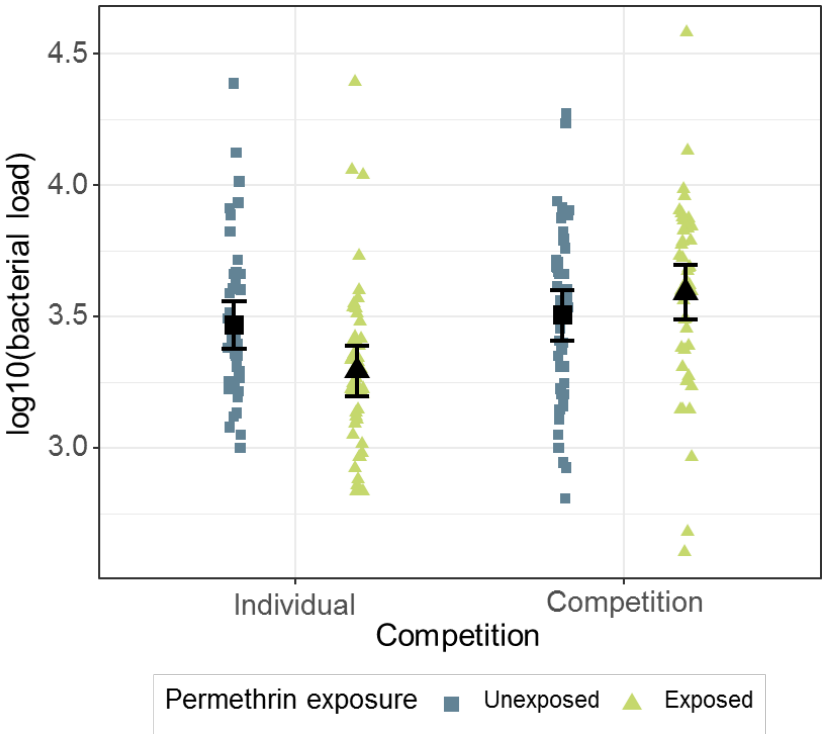

Figure 3. Number of bacteria (log10 transformed) recovered in mosquitoes injected with living *E.coli*, after 24 hours of incubation. N=187. Error bars show the 95% confidence intervals of the means.

4.5 Discussion

Insecticide residues are frequently found in mosquito breeding sites, especially around agricultural areas. We show that even at low concentration in water, permethrin can affect the life-history of the malaria vector *Anopheles gambiae* and, in particular, enhance the efficacy of its immune response

once it emerges as an adult. However, several of these effects were less apparent if mosquitoes had competed for food during their development.

Mosquitoes that reared in groups and thus forced to compete for food responded as expected (87, 131): they were more likely to die as juveniles, pupated later, and developed into smaller adults than individually reared mosquitoes. They also had a weaker immune response: they were less likely to melanize beads, deposited less melanin on the beads, and were less able to slow the replication *E.coli.* This corroborates the suggestion that the mosquito's immune responses are energetically costly, so larval undernourishment limits the mosquitoes' ability to defend themselves against pathogens (265, 266).

Mosquitoes that were exposed to a low dose of permethrin were also more likely to die and pupated later, but they developed into slightly larger adults than unexposed larvae, as found for *Aedes aegypti* exposed to malathion (267) or spinosad (60). Whereas in these studies the death of some larvae reduced competition for food and thus enabled survivors to grow larger, the effect in our study was found for individually reared mosquitoes. We therefore suggest that permethrin is more likely to kill the smallest, weakest larvae, while the larger ones are more likely to survive to become adults.

That larvae exposed to permethrin developed stronger immune responses as adults could be explained in at least two ways. First, as just discussed the exposure could let only the strongest individuals, so those with the most effective immune responses survive. Second, detoxifying insecticides may use some mechanisms that are also involved in the immune response. For example, exposure to insecticides leads to the long-lasting expression of genes, as defensin, that are involved in the mosquito's immune responses (267). Furthermore, several studies reported positive associations between insecticide metabolic resistance and immunocompetence. For example, the activity of phenoloxidase, an enzyme that plays a central role in melanization (140) and contributes to antibacterial defense (136), is greater in resistant *Culex pipiens* mosquitoes (137) and in resistant *Plutella xylostella* moths (138) than in sensitive ones. Also, several antimicrobial peptides (AMP) are expressed to a greater degree in resistant *Culex pipiens* and *Anopheles gambiae* (220, 268), and nitric oxide synthase is expressed more in resistant *Anopheles stephensi* (269).

That mechanisms are shared could, in turn, be explained in two ways. Genes that are involved in detoxifying insecticide can have a pleiotropic effect on immune responses. This may indeed be the case for phenoloxidase, which is not only necessary for the melanization immune response, but may also play an active role in insecticide detoxification (138). Alternatively, or additionally, a third physiological process could link detoxification and immune responses. One possibility is an interplay with reactive oxygen species, for permethrin induces a high level of oxidative stress (270), which in turn increases melanotic encapsulation (154) and antibacterial response (150).

Despite the link between the detoxification of insecticides and the immune responses, and contrary to our expectation, most effects of permethrin were not apparent if larvae had been forced to compete than if they had been reared individually. This suggests that energetic resources play a central role in the mechanisms involved. This could be a direct consequence of limiting resources; when food is limited, pleiotropic effect may simply not be possible. It could also implicate the role of oxidative stress; with less food available, less energy is metabolized and less oxidative stress is generated, which would weaken the link between detoxification and immune responses

In summary, we showed that the presence of sublethal dose of insecticide in the larval environment was associated with higher immunocompetence for two traits measured: melanization and antibacterial response. However, most of the beneficial effects observed disappeared if mosquitoes were put in competition for food during their development. Thus, larval competition probably induces strong energetic constraints that limit the activation of immune mechanisms consecutive to an insecticide exposure. Together, our results show that insecticides in larval environment can have important carry-over effects on important mosquito defenses mechanisms related to vectorial capacity. Moreover, the obtained results also underline the importance of larval competition for food – ubiquitous in nature – in shaping the response of mosquitoes to insecticidal stress.

Chapter 5

Exposure to an insecticide during development primes *Anopheles gambiae* adults for higher antioxidant capacity

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5.1 Abstract

Early-life stress can have long-lasting consequences on traits such as growth, survival, reproduction, or immunocompetence. Since oxidative stress, which results from an imbalance between pro-oxidants and antioxidant, may underly such traits, we aimed to understand how developmental stress can impact adult's ability to cope with oxidative stress. Further, we aimed to link any differences in oxidative stress and antioxidant capacity to variation in immunity in adults. We therefore reared *Anopheles gambiae* mosquitoes either singly or under competition and exposed them as larvae or adults to a sublethal dose of a pyrethroid insecticide, and assayed how these stress factors affect the oxidative state in adults. From each mosquito, we measured several antioxidant and oxidative damage markers, as well as the melanization response as a proxy of immunity. We found that early-life exposure to the insecticide increased adults' antioxidant capacity, and that a higher antioxidant defence was associated with an increased efficacy of the melanization response. However, if larvae were reared in competition the increase in antioxidant capacity was only apparent if the adults were re-exposed to the insecticide. This suggest that the extent to which individuals are primed by early-life stress could be limited by access to resources (which is here limited by larval competition). Our results show that such transstadial stimulation by the insecticide may prepare the organism to face further oxidative challenges later in life, which in turn could affect other fitness related traits like immunity.

5.2 Introduction

Environmental stress can impact an individual's fitness in several ways (271, 272), and thus it is considered as a major driving force of evolution (273–275). In order to mitigate the effects of stressors like, for example, pathogens, temperature variation, and xenobiotics, organisms have evolved immune responses (276), heat-shock proteins (277), or xenobiotic detoxifying enzymes (278, 279). While stress often leads to immediate changes in an organism's phenotype, it can also have long-lasting effects. Stress early in life can thus have profound effects on traits such as longevity, reproduction, or immunocompetence much later in life (280, 281). In mosquitoes for example, the temperature during larval development impacts adult size (87, 94, 282), longevity (94, 283), fecundity (284–286), and immunity (82), including the mosquitoes' competence for human pathogens (82, 214, 225, 282). Similar effects on adult mosquitoes have also been documented after developmental nutritional stress (86, 88, 89, 226, 287) and exposure to insecticides (53, 55, 119, 191, 194).

That a larval stressor induces changes in several adult traits may be explained by two non-exclusive hypotheses. First, early-life stress might intensify the trade-offs faced by an individual by constraining the resource allocation between the different life-history traits (288). For example, larval nutrition affects the prevalence of malaria in the mosquito *Anopheles gambiae*, and this effect is enhanced when larvae are exposed to a larvicide (95). Second, the affected traits may be related to one another by shared mechanisms or via pleiotropic effects. Thus, the expression of heat shock proteins – which is induced by various biotic and abiotic stressors (277) – has pleiotropic effects on insect longevity, fecundity (289, 290), and immune responses (291). Similarly, stress-induced secretion of insect neurohormones may have repercussions on many aspect of insect physiology (292), including reproduction (293) and immunity (294).

Oxidative stress (OS) is a further trait that is induced by several environmental stressors, such as heat (295), food deprivation (296) and insecticides (155) and that is implied in several physiological mechanisms. OS, which results from the imbalance between pro-oxidants and antioxidants leads to the oxidative damage of, e.g., proteins, DNA, and cell membranes (297). The release of reactive oxygen species (ROS) is a direct consequence of aerobic metabolism, and is therefore involved in virtually every energy-demanding physiological mechanism (298). It has therefore been suggested that OS constrains the evolution of several life-history traits (143, 146). However, OS is not only detrimental; rather it also plays an important role in, for example, the innate immune response, where ROS are directly used to kill pathogens (150–153), act as immune signaling molecules (148), and modulate the ability of insects to melanize a parasite (154).

One class of insecticides that increases OS is the pyrethroids (155, 242, 270). These are widely used and are now ubiquitous in freshwater (182, 183), where they can affect aquatic organisms including mosquito larvae. Because pyrethroids are also the only class used in insecticide-treated bed-nets for

the control of malaria, mosquitoes may be exposed at different life stages with consequences on their behavior and physiology (e.g. (46, 55, 299)). However, whether an early-life sublethal exposure to pyrethroids has long-term consequences on mosquitoes' oxidative state has, to our knowledge, not been investigated yet. Importantly, given the close association between OS and immunity, changes in OS due to an insecticide exposure might affect the insect's immune responses.

In the present study, we used *Anopheles gambiae* to assay how an early-life exposure to a pyrethroid insecticide affects adult OS, and whether differences in OS covary with the mosquitoes' melanization immune response. Melanization is involved in wound healing (300) and melanotic encapsulation of pathogens (140, 301, 302). A previous study using a mosquito strain that is under chronic OS found that melanization is stronger than in a strain with a normal physiological state (154). However, how oxidative balance is plastically regulated after an insecticide exposure and how it affects immunity in an unselected strain of mosquito is unclear. Additionally, we also tested whether larval intraspecific competition would intensify any trade-off associated to OS defenses and immunity, by limiting the access to resources. Larval nutrition affects the melanization response of mosquitoes (255), and metabolic rate during and after starvation periods strongly affects the antioxidant activity of damselflies (303), but how larval nutrition interacts with insecticide exposure to affect oxidative stress and melanization has, to our knowledge, never been assessed.

5.3 Material and Methods

We assessed the oxidative balance and its association with the melanization response of adult mosquitoes using mosquitoes of the insecticide-sensitive Kisumu strain (166) that were experimentally exposed to permethrin at larval and/or adult stages. Adults from the different treatments were first micro-injected with a Sephadex™ bead in the thorax, then kept for 24 hours before their abdomen was homogenized and three OS markers quantified. Finally, we dissected the thorax of each individual to recover the injected bead, and the amount of melanin deposited on each bead measured.

All experiments involving living mosquitoes were run in an insectary maintained at 26.5 ± 0.5 °C, 70 ± 5% humidity and a 12:12 light to dark photoperiod. One exception is the bead injection manipulation, which has to be done at room temperature.

5.3.1 Determination of sublethal dose of permethrin

The dose used for the experiment was the same as the dose used in Chapter 4. Briefly, we tested five different concentrations of permethrin for their lethality on *An. gambiae* larvae: 0.04, 0.1, 0.15, 0.3 and 0.8 µg/L. Controls had a solution with 0.03% volume per volume ethanol (236 µg/L). This concentration of ethanol was not found to induce significant mortality (tested against a mortality of 0 using a 2-sample test for equality of proportions (*prop.test* in R)). Based on the results, we chose to use permethrin at a final concentration of 0.1 µg/L for the following experiments, as such sublethal

concentration was the highest tested before permethrin induced significant mortality. The concentration of ethanol used as control was fixed at 0.01% v/v (78.9 µg/L).

5.3.2 Mosquito rearing and exposure to permethrin

Freshly hatched An. gambiae larvae (0-3h old) were put into glass petri dishes (4 cm diameter) containing 4mL of 0.01% ethanol supplemented with 0.1 µg/L or no permethrin. Larvae were reared either individually or, to induce among-larvae competition, in groups of three. Tetramin Baby® fish food was provided daily according to the age of the larvae: 0.04, 0.06, 0.08, 0.16, 0.32 and 0.6 mg/larva at age 0, 1, 2, 3, 4, and 5 or more days, respectively [33]. Each petri dish was provided with an amount of food calculated for 1 larva, which implies that, on average, larvae from the competition-treatment received about one third of the food received by individually reared larvae. Pupae were transferred individually to 50mL Falcon™ tubes. If more than one female emerged from a petri dish where a group of three had been competing, we selected one randomly for further analysis to guarantee independence of data. The selected females were transferred to 21 x 21 x 21 cm cages according to their treatment and age (one cage per day of emergence and treatment), where they had constant access to a 6% sucrose solution. Four days following the emergence, females were exposed for 2 minutes to permethrin 0.75% treated papers (*exposed adults*), or pyrethroid control papers (*unexposed adults*) following WHO exposure protocol (199). Mosquitoes were then kept in cages until further manipulation.

5.3.3 Bead injection

The injections were performed 2 days after the adults were exposed to permethrin or control papers (i.e. 6 days old), following the protocol described in Barreaux et al. with minor modifications (262). Briefly, females were anesthetized on ice and injected in the thorax with a negatively charged carboxymethyl Sephadex® C-25 bead (Sigma-Aldrich Inc., St. Louis, Missouri) using glass microcapillaries. Injected females were transferred in cages kept at insectary conditions with constant access to 6% sucrose solution. After 24 hours, cages were put at -20°C for a few minutes, and anesthetized mosquitoes were transferred individually in 1.5 mL microcentrifuge tubes. The tubes were put at -80°C to kill and preserve mosquitoes.

5.3.4 Sample preparation for oxidative stress measurements

Mosquito samples were prepared in small groups while being kept on ice during manipulation to prevent any degradation. For each mosquito, the abdomen was first separated from the thorax using micro-forceps, and the thorax was then kept at -20°C until dissection for the bead. The abdomen was weighed to the closest µg, put in a new 2 mL microcentrifuge tube with 80 µL of PBS pH 7.2. A 5 mm diameter autoclaved steel bead was then put in the tube, and the abdomen was homogenized using a

tissue lyser at a frequency of 45 Hz for 4 minutes with an ice-cold metal drum. The resulting homogenate was centrifugated at 9'300 rcf for 10 min at 4°C, and the supernatant was then transferred in new 1.5 mL microcentrifuge tubes for the analyses of oxidative stress markers. All aliquots as well as the remaining supernatant were finally kept at -80°C until they were assayed.

5.3.5 Oxidative stress markers

For each single mosquito's abdomen, we quantified: 1) glutathione (both its reduced (GSH) and oxidized (GSSG) states), a non-enzymatic antioxidant frequently used as a marker of cellular oxidative stress (243), 2) superoxide dismutase (SOD) activity, an important antioxidant enzyme that scavenge superoxide anions and catalyze their dismutation into molecular oxygen or hydrogen peroxide (304), and 3) malondialdehyde (MDA), which is a by-product of lipid peroxidation and used as an indicator of oxidative stress level (305, 306). In addition to the total glutathione amount, the ratio between its reduced (GSH) and oxidized (GSSG) state was also used in the analysis as a cellular oxidative stress marker (307).

Glutathione

Glutathione extraction and determination was performed immediately after tissue homogenization following Rojas Mora et al. (308) with minor modifications. Briefly, 110 µL of a mix of formic acid (Sigma-Aldrich Inc. St-Louis, Missouri), glutathione reduced ethyl ester (internal standard, Sigma-Aldrich Inc. St-Louis, Missouri) and ultra pure water was added to the 15 µL of homogenate. Final concentrations were 0.058% for formic acid, and 50 ng/mL for the glutathione reduced ethyl ester. Samples were then centrifugated for 15 minutes at 9'300 rcf, and 115 µL of the supernatant were filtered with a 22 µm PTFE filter (BGB, Germany) into an HPLC glass vial. Glutathione was finally quantified with an HPLC-MS.

Superoxide dismutase (SOD)

To quantify SOD activity, we followed standard procedures of a commercial kit (Cayman, USA) with minor modifications. Briefly, the abdomen homogenate was first diluted 1:8 in PBS before it was used as described in the kit protocol. Samples were run in duplicates with an average within-plate coefficient of variation (CV) of 7.9%. Further, 42 samples were assayed in different plates, showing high inter-plate repeatability (r = 0.88).

Malondialdehyde (MDA)

MDA was quantified using the procedure described in Mendonça et al. (309) with minor modifications. First, 40 µL of NaOH 1.2 M were added to 8 µL of abdomen homogenates, and samples were incubated at 60°C for 30 min for protein hydrolysis. Samples were then cooled down for 2 minutes at 4°C, and 10 µL of the internal standard (d2-MDA 30 µM in 0.1 M HCl) were added. Previously hydrolyzed proteins were precipitated by adding 142 µL of trichloroacetic acid 20%, samples were centrifugated for 5 min

at 9'300 rcf, and 180 µL of supernatant were transferred in a new 1.5 mL microcentrifuge tube. MDA was then derivatized by adding 18 µL of 2,4 dinitrophenylhydrazine 5mM and by incubating the samples for 10 minutes at room temperature with gentle agitation. Sample pH was then alkalized by adding 22 µL of NaOH 10 M, and two phase-to-phase extractions were performed by adding 250 µL of a solution of toluene and cyclohexane (1:1 v/v) and transferring the supernatant to a new 1.5 mL microcentrifuge tube. The recovered organic phase was evaporated in a SpeedVac ® at 35°C, and the pellet was reconstituted in methanol 50%. The extracts were finally filtered with a 22 µm PTFE filter (BGB, Germany) into an HPLC vial before being analyzed with an HPLC-MS. A standard curve, ranging from 0 to 40 µg/mL, was done with MDA tetrabutylammonium salt (Sigma-Aldrich Inc. St-Louis, Missouri), and all the standards went through the extraction protocol together with the samples.

5.3.6 Bead recovery and melanization measurement

Mosquitoes that were not prepared for OS analyses and the thorax of mosquitoes that did were all dissected in a 0.1% methyl green saline solution, and a picture of each bead was taken under a microscope at 20x magnification. Melanization was determined in two ways. First, each bead was visually assessed to discriminate fully unmelanized from melanized beads (binary descriptor). Second, the amount of melanin of melanized beads was quantified (continuous descriptor). To quantify melanization, we used the ImageJ software v1.51 (198). Parts of the beads that were not covered with melanin were first filtered (based on the color spectrum of unmelanized parts) and made white. Then, the *mean gray value* of each bead picture was extracted and used as a melanization index for the statistical analyses. A gray value of 0 means that the bead is entirely white and a value of 256 indicates a totally black bead. Finally, ImageJ was also used to measure bead diameter in µm.

5.3.7 Statistical analyses

All analyses and figures were done in R v 3.6 (200). Significance was assessed with the *Anova* function from the *car* library (201), using a type III sums of squares if an interaction was significant or a type II sums of squares otherwise. Unless otherwise stated, explanatory variables were always tested for their interactions, and non-significant interactions were removed from the final models. No other model selection was performed. For linear models (LM), normality of residuals was visually checked and homoscedasticity was tested using a Breusch-Pagan test (*bptest* function of the *lmtest* library (263) in R).

In cases of significant interactions, contrast analyses were done between the factors of interest using *emmeans* (computing Estimated Marginal Means (EMM)) and *pairs* functions of the *emmeans* library in R, with p-values being adjusted using the *mvt* method.

Larval development and body size

Larval mortality was analyzed using a Generalized Linear Model (GLM) with quasibinomial errors. The response variable was mortality in each petri dish, and the model was weighted by the number of larvae initially put in each petri dish. Explanatory variables were larval exposure to permethrin and larval competition.

We tested whether larval developmental time, measured as the number of days from hatching to pupation, was affected by permethrin exposure and/or larval competition by using a Cox's proportional hazard model implemented in the *coxme* library in R (264). Larvae that died before pupation were censored in the analysis. Similarly, in the competition treatment, if one or several larvae died, the entire petri dish was censored as competition for the remaining larvae was reduced. For petri dishes from the competition treatment in which none larvae died, the average developmental time was used for the analysis, while the model was weighted by the number of larvae initially put in the petri dish.

Finally, we used a Linear Model (LM) to test for the effects of competition and larval exposure to permethrin on body size inferred from the wing length.

Post-injection survival

We tested whether the proportion of mosquitoes that died within 24 hours following the injection was differed across treatments using a GLM with a binomial distribution of errors. Explanatory variables were larval exposure to permethrin, larval competition, and adult exposure to permethrin.

Melanization

We analyzed the immune response in two different ways. First, we performed a generalized linear model (GLM) with a binomial distribution of errors to test the melanization success (the proportion of mosquitoes that partially or completely melanized their bead) across treatments. Explanatory variables were larval exposure to permethrin, larval competition and adult exposure to permethrin. Second, we used the bead grey value to test whether the amount of melanin deposited on the beads differed across treatments. Bead grey value was first rescaled in a way to have values bounded between 0 and 1, and then values were logit-transformed to meet normality of the residuals. We then performed a linear model to test whether larval exposure, larval competition and adult exposure, and affected the bead grey value. Finally, bead diameter was added as a covariable in both analyses.

Oxidative stress

We performed a principal component analysis (PCA) using: 1) the total amount of glutathione (oxidized + reduced), 2) the GSH:GSSG ratio, 3) the activity of SOD, and 4) the amount of MDA. From the loading values of the PCA we selected a principal component that reasonably put oxidant marker (MDA (square-root transformed)) in opposition with antioxidant markers (SOD, total glutathione or GSH:GSSG ratio (all log10 transformed)). While PC1 explained 41.4% of the variance and PC2 27.2%,

the latter was more coherent as an OS indicator as explained above (loadings: *ratio GSH:GSSG:* -0.03; *total glutathione:* -0.51; *SOD:* -0.83; *MDA:* 0.23). The eigenvalue of PC2 was of 1.09. The details of the PCA for each principal component are available as **Supplementary Information**; **Tables S1** and **S2**.

We hence used PC2 (hereafter oxidative state) for three different analyses. A high PC2 indicating high oxidative stress – higher MDA levels and lower GSH:GSSG ratios -, while low PC2 value indicating a higher antioxidant defense. First, we used a robust linear model (rLM) to assess the impact of the treatments (larval exposure to permethrin, larval competition, adult exposure to permethrin) on the oxidative state of the mosquitoes. Robust linear models allows to deal with variance heterogeneity (310), as implemented in the *rlm* function from the *MASS* package (311). In the second and third analyses, we tested whether mosquito oxidative state was correlated with their ability to melanize beads. We built an LM with bead grey value as the response variable, and oxidative state (PC2) as an explanatory variable. Finally, we used a GLM with a binomial distribution of errors to test whether oxidative state affected bead melanization success. In these two latter models, larval competition, larval exposure, adult exposure and bead size were added as a covariables.

5.4 Results

5.4.1 Larval development

In total, 1475 larvae were reared (503 individually, 972 in competition). Mortality ranged from 2.9% (95% CI: 1.1 to 7.3%) to 6.9% (95% CI: 4.4 to 10.8%) and was increased by the presence of permethrin in water (X^2=6.80, df=1, p=0.009). In average, unexposed larvae experienced 3.5% (95% CI: from 2.1 to 5.9%) mortality against 6.4% (95% CI: from 4.4 to 9.1%) for permethrin exposed larvae. There was no effect of competition (X^2=0.87, df=1, p=0.35) and no interaction between both stressors (X^2=0.02, df=1, p=0.87)

Development time (from hatching to pupation) ranged from 7 to 13 days. In average, it took 7.95 ± 0.03 (mean ± 95%CI) days to pupate for individually reared larvae, against 10.2 ± 0.05 days for larvae reared in competition (X^2=972.19, df=1, p<0.001). Larval exposure to permethrin had no global effect (X^2=1.65, df=1, p=0.20), but there was a significant interaction between competition and larval exposure (X^2=5.11, df=1, p=0.024). The analysis of contrasts showed that permethrin exposure slightly increased the development time of individually reared larvae (from 7.90 ± 0.04 days to 8.01 ± 0.05; z=2.40, p=0.016) but had no effect on the development time of larvae reared in competition (from 10.24 ± 0.08 days to 10.17 ± 0.07 days respectively, z=-1.0, p=0.32).

5.4.2 Wing size

Larvae reared in competition resulted in smaller adults than those reared individually (wing length: 2.83 ± 0.02 mm against 3.10 ± 0.01 mm respectively; $F_{1,318}$=446.02, p<0.001). Larval exposure to

permethrin did not affect wing size ($F_{1,318}$=1.33, p=0.25), and there was no interaction between both factors ($F_{1,317}$=0.03, p=0.87).

5.4.3 Bead injection

A total of 417 mosquitoes were injected, of which 83 died in the first 24 hours after the injection (19.9% mortality, (95% CI: 16.3 to 24.0%)). Mosquitoes reared in competition experienced a three-fold increase in mortality following the injection compared to individually reared mosquitoes (competition: 30.2%, (from 24.4 to 36.7%); individual: 9.3%, (from 6.0 to 14.0%); X^2=29.11, df=1, p<0.001). There was also a global negative effect of larval exposure on post-injection survival (unexposed: 17.1% (12.5 to 22.8%); exposed: 22.6% (17.5 to 28.7%); X^2=7.36, df=1, p=0.007) but no effect of adult exposure to permethrin (X^2=1.70, df=1, p=0.19). Moreover, their interaction was significant (X^2=8.03, df=1, p=0.005). Contrast analysis found that adult exposure to permethrin increased mortality of mosquitoes that have already been exposed as larvae (z=2.79, p=0.005), but not that of mosquitoes that were not exposed previously (z=-1.22, p=0.22). No other interaction was significant (all p>0.42).

5.4.4 Melanization

Beads were recovered in 285 out of 334 mosquitoes. We found that 213 out of these 285 beads were entirely or partially melanized (76.1% (95% CI: 70.9 to 80.7%)). There was no effect of larval exposure (X^2=0.49, df=1, p=0.48), larval competition (X^2=0.0, df=1, p=0.96) or adult exposure to permethrin (X^2=0.50, df=1, p=0.48). Nevertheless, we found a trend for a triple interaction between all three factors (X^2=3.59, df=1, p=0.058; **Fig. 1**). Other interactions were not significant (p>0.14). Due to the number of levels involved in the triple interaction and the relatively small effect sizes, the post-hoc analysis did not find any difference between treatments (pairwise comparisons performed separately for individual and competition treatment; all p>0.244).

Mosquitoes that successfully melanized their bead were also tested for the amount of melanin they deposited around it (bead mean grey value). We found that there was a trend for a lower amount of melanin on beads from mosquitoes reared in competition ($F_{1,212}$=2.88, p=0.091, **Fig. 2**), but no effect of larval exposure ($F_{1,212}$= 0.21, p=0.65), or adult exposure ($F_{1,212}$=0.08, p=0.78). Further, none of the interaction was significant (all p>0.20).

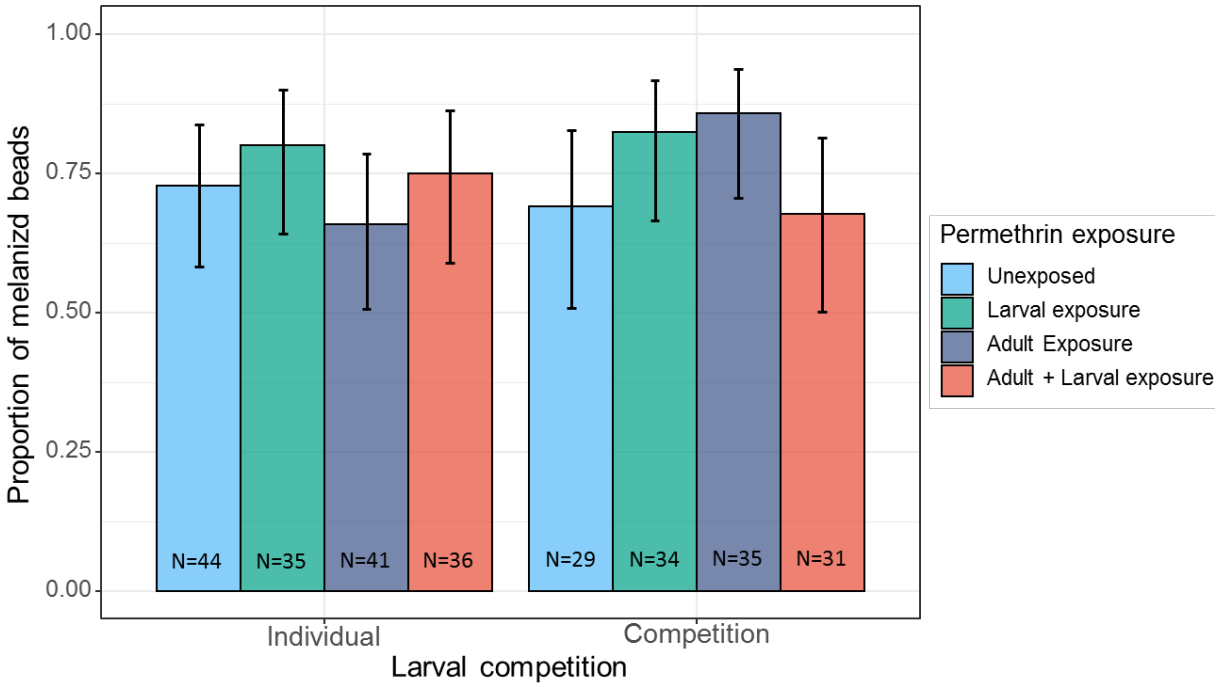

Figure 1. Melanization success of mosquitoes injected with a Sephadex™ bead. Shown are the proportion of mosquitoes that successfully melanize – partially or completely – their bead in function of larval competition (left or right panel) and the exposure of larvae or adults to permethrin (color legend). Error bars show the 95% confidence intervals.

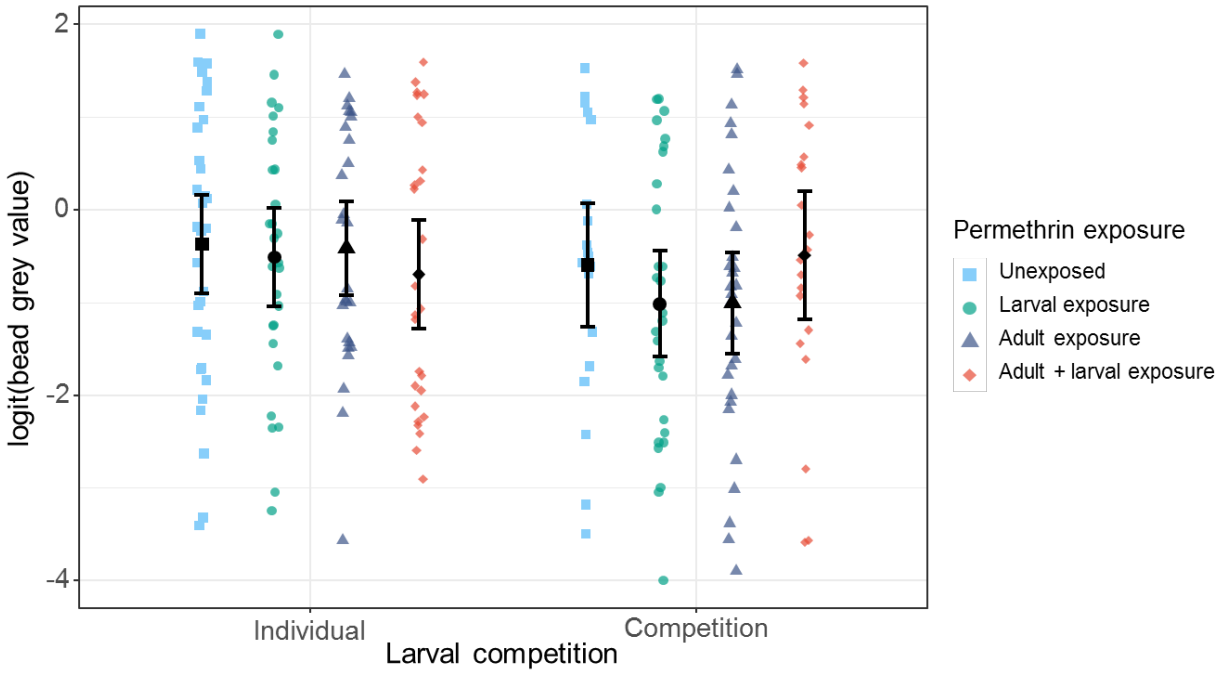

Figure 1. Amount of melanin deposited on the injected beads. Shown is the mean bead grey value (scaled and logit transformed) used as a proxy for the amount of melanin, in function of larval competition and the exposure of larvae or adults to permethrin (color legend). N=213. Error bars show the 95% confidence intervals.

5.4.5 Oxidative stress

We found that permethrin exposure at larval ($F_{1,227}$=9.86, p=0.002) or adult stage ($F_{1,227}$=16.19, p<0.001) led to a decreased mosquito oxidative state (e.g. decreased oxidative damage), but that larval

competition had no global effect on the oxidative state ($F_{1,227}$=0.42, p=0.52). However, we found significant interactions between larval and adult exposures ($F_{1,227}$=9.09, p=0.003), adult exposure and larval competition ($F_{1,227}$=15.06, p<0.001), as well as the triple interaction between all the treatments ($F_{1,227}$=4.86, p=0.008; **Fig. 3**). A post-hoc analysis (performed separately for individually reared or competing mosquitoes) showed that in individually reared mosquitoes, the lowest oxidative state is reached in mosquitoes exposed to permethrin at larval stage or at both larval and adult stage. In mosquitoes reared in competition however, larval exposure did not decrease oxidative state anymore, while an exposure at both larval and adult stage still did. Adult exposure alone, irrespective of the presence of competition, appeared to have no effect on the oxidative state of the mosquitoes.

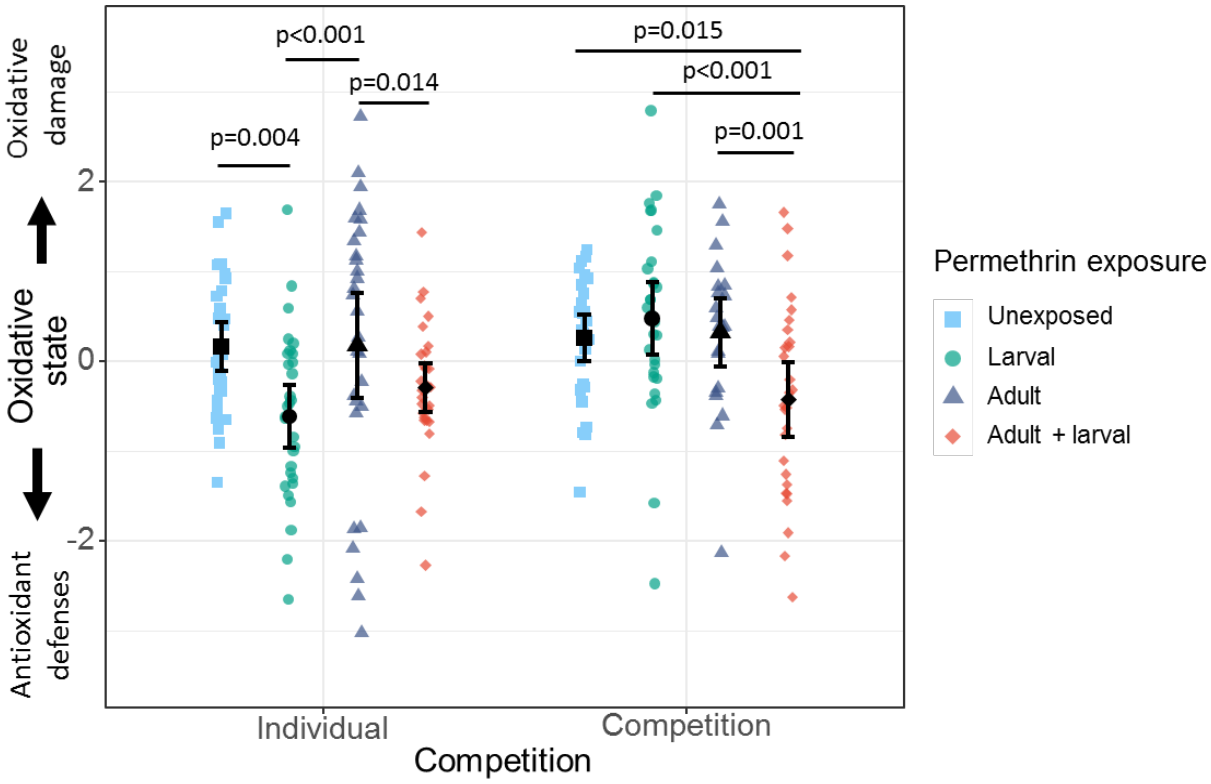

Figure 2. The graph shows the differences of oxidative state in function of the presence of larval competition and the exposure of larvae or adults to permethrin (color legend). N=227. Error bars show the 95% confidence intervals of the mean.

We also found that oxidative state was negatively correlated with the amount of melanin deposited on beads ($F_{1,139}$=10.40, p=0.002; **Fig. 4**). However, no association was found between the oxidative state and melanization success (X^2=1.37, df=1. P=0.24).

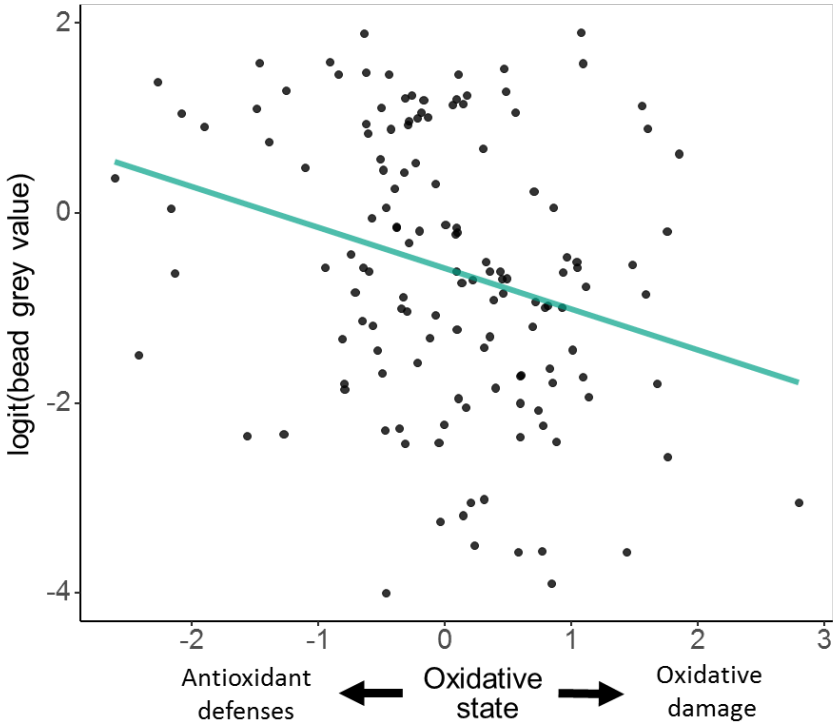

Figure 3. Correlation between bead mean grey value (scaled and logit transformed), use as a proxy for the amount of melanin deposited by mosquitoes around the bead injected in their thorax, and the oxidative state. N=140.

5.5 Discussion

In the current study we show that early-life stress impact adult oxidative state, and this might have consequences for mosquito immunity. In particular, our results showed that exposure to permethrin during development increased the antioxidant response of adult mosquitoes (**Fig. 3**), suggesting early-life stressed primed mosquitoes to be more resilient to OS later in life. Also, in contrast to what was predicted by the results of a previous study (154), we found that higher melanization was achieved by mosquitoes with an increased antioxidant defenses (**Fig. 4**). As melanization itself induces OS (312), our result suggest that mosquitoes' antioxidant defense may be an important constraint of their melanization capacities.

5.5.1 Effect on permethrin on development

Insecticide-exposed larvae had an increased developmental time when individually reared, and experienced a slightly higher mortality (by ca. 3%). However, adult body size was not affected by larval exposure to permethrin. These results confirm that permethrin at a sub-lethal dose constrained the development of mosquito larvae, but also show that mosquitoes can fully compensate for the early-life perturbation by taking slightly more time to complete their development.

5.5.2 Oxidative state in adult mosquitoes

We found that larval competition and larval/adult exposure to permethrin interact in complex ways to affect mosquitoes' oxidative state. First, we found that competition alone did not induce any change in adult oxidative state. Thus, while it is known that transient food deprivation has short-term consequences on the oxidative balance (303), our results show that nutritional stress during development did not impact OS in adults. Possibly, the oxidative state may be restored when food level is not limited anymore (i.e. at adult stage). In our study however, it cannot be excluded that the absence of effect of competition was somewhat hidden by the higher mortality observed in this treatment following the injection.

Second, despite pyrethroid detoxification is known to induce strong oxidative stress (155), we unexpectedly found that permethrin exposure did not seem to increase the levels of OS. However, it should be considered that OS measurements were done following an injection. As the injection itself causes a wound that led to a ca. 20% mortality after 24 hours, our OS measurements might be affected by the wound and its consequences for the organism (e.g. healing process, immune responses, and melanization). Thus, our measures of OS do not necessarily represent mosquitoes' basal state, but more likely their response to the initial wound. Here, we found the antioxidant response to be stronger when mosquitoes were previously exposed to permethrin at larval stage (individually reared mosquitoes) or at both larval and adult stages subsequently (competing and individually reared mosquitoes; **Fig. 3**).

Previous studies have already shown that developmental stress could prime individuals to have a better antioxidant capacity (or OS tolerance) later in life. For example, it was shown in zebra finches that a mild heat-stress during development induced a higher antioxidant response when they were exposed to a second heat stress as adults (295). Our results suggest that larval exposure to the insecticide had a priming effect on the antioxidant defence in adult mosquitoes. Indeed, as pyrethroid exposure induces high OS (155), a strong antioxidant response is expected to rapidly follow to limit and repair any OS damage caused by the insecticide. This antioxidant defense may have primed mosquitoes, and thus adults responded more efficiently to the OS induced by the wound healing and the melanization response. In our case however, no positive effect on the antioxidant response was detected when mosquitoes were only exposed at adult stage, suggesting that priming effect is, in this case, essentially triggered during larval stage.

A more complex result is the fact that neither larval exposure nor adult exposure alone did increase the antioxidant response of mosquitoes reared in competition: OS was only reduced when mosquitoes were exposed at both larval and adult stages. One possibility is that the antioxidant stimulation induced by larval exposure may be energetically costly, and its long-term effect may be reduced when resources are limited. That larval and adult exposures together still reduced oxidative stress in

mosquitoes reared in competition suggests however that repeated sublethal exposure contributes to increase further response to oxidative stress. The potential for repeated stresses to increase an initial stimulatory – or priming – effect has also been described in heat-stressed *Caenorhabditis elegans* nematodes (313) and after repeated hydrogen peroxide exposure in both mammal cells and in *Drosophila melanogaster* flies (156). That the strength and duration of a – presumably energetically costly – priming effect depends on whether stress is repeated in time may therefore be an adaptive mechanism to prevent these costs to have deleterious repercussions on other life-history traits it may trade-off with. However, to this stage we cannot rule out other possible effect from competition, and further studies are required to test whether is solely resource limitation that constrains the extend to which individuals are primed or whether other physiological traits are involved.

5.5.3 Oxidative state and melanization

High levels of antioxidants – mostly GSH – may inhibit melanogenesis by preventing the melanotic cascade to be triggered (314). Thus, individuals might need to expose themselves to OS in order to enhance the melanization response (315, 316) by reducing the antioxidants that quench various precursors of melanin, as phenoloxidase or tyrosinase (314). For instance, such prediction is supported by a previous study on *Anopheles* (154), where it was shown that a higher melanization response was achieved by a mosquito strain that is in a chronic OS state. In contrast, we found that mosquitoes that had a higher antioxidant response had a better melanization capacity (**Fig. 4**). A major difference between Kumar's study and ours is the moment at which oxidative state was measured. In their experiment, oxidative stress markers and melanization were assessed separately using different mosquitoes, which allowed them to estimate their basal oxidative state. On the opposite, our measurements were done on mosquitoes that were previously injected. Thus, while they found that a high basal level oxidative stress can increase mosquitoes' melanization capacity, which is in line with the mechanisms involved (discussed above), our results suggest that beads are ultimately best melanized by mosquitoes capable of a high antioxidant response. In line with this hypothesis, Kumar et al. found that the mosquito strain they tested had a 3-5 fold increased activity of antioxidants (SOD and catalase) following a blood meal compared to a sensitive strain. As taking a blood meal also induces OS (150), their results actually suggest that the mosquito strain they used in that study not only had a constantly high level of OS, but was also able to trigger a strong antioxidant response after an OS-challenge. The results of both studies are therefore most probably complementary and not in contradiction: a high basal oxidative state may help mosquitoes to melanize, but their antioxidant capacity may ultimately determine the extent to which they can do so without suffering from excessive oxidative damages.

A possible limitation of the proposed role of antioxidant in the melanization process (i.e. protection against oxidative damage) lies in the observed mortality following the injection. Indeed, mosquitoes that were exposed at both larval and adult stages showed a lower oxidative stress but suffered from higher mortality than those that were exposed at one of the two stages only. It is therefore possible that the antioxidant response has an additional unknown function in the melanization process in addition to protecting the organism against oxidative damages. Alternatively, it may be that oxidative damages following the injection only poorly contributed to the death of these mosquitoes, and that repeated insecticidal stress at larval and adult stage may impose some costs (e.g. related to the stimulation of the antioxidant response or to detoxifying mechanisms) that impedes their survival to the injury.

5.5.4 Implications of the results and future research

Because both the oxidative balance and melanization pathway are involved in several phenotypic traits, the results found are likely to have broader implications than those previously described.

For example, because oxidative damages induced by reproduction have been proposed as a proximate mechanism underlying the reproduction-survival trade-off (143, 144), any increase in the antioxidant capacity is eventually expected to alleviate the physiological costs of reproduction, and may thus contribute to both an increased reproduction success and prolonged life span. Supporting this hypothesis, a study shown that the administration of antioxidant restored the age-related loss of fecundity in 2 strains of *Anopheles gambiae* (145). What is not clear yet however, is to which extent the observed benefits of a sublethal insecticidal stress overcome its potential costs. In particular, our results suggest that the benefit-cost ratio is probably strongly dependent of resource availability.

Another important property of the antioxidant response is its implication in insecticide tolerance. It is known that a higher antioxidant activity helps mosquitoes to survive pyrethroid exposure (147, 317), and an increased expression of antioxidant is often observed in laboratory or field resistant mosquito strains (147, 210, 318). Our results therefore indicate that mosquitoes exposed at sublethal concentration of permethrin may better survive to further exposure via a stimulation of their antioxidant defences. Such plastic increase in insecticide tolerance has indeed been shown with different xenobiotics and a pro-oxidant (hydrogen peroxide) (121–123). But so far, these effects were mainly explained by an increase in detoxifying enzymes as cytochromes P450 (124, 125, 193). Our results suggest that mosquitoes' antioxidant defence may be a complementary mechanism through which tolerance to xenobiotics may be plastically increased. An interesting example of the importance of antioxidants in pyrethroid resistance is the glutathione-s-transferase enzyme, which has an important role in the detoxification of various insecticides (319). Glutathione-s-transferase was found to be increased following pyrethroid exposure (320, 321), but is now considered to provide pyrethroid

resistance thanks to its antioxidant but not detoxifying property (317, 322). Interestingly, if both mechanisms – increased detoxifying enzyme activity and antioxidant response – can be plastically induced, mosquitoes exposed to a sublethal dose of insecticide acquire similar properties than metabolically resistant mosquitoes (210, 219). This may be an important mechanism to consider in vector or pest control strategies where insecticide residues are expected to be found in the environment.

To conclude, we showed in the present study that an exposure to a sublethal dose of permethrin induces a long-lasting stimulatory effect on mosquitoes' antioxidant defence, allowing them to better respond to oxidative challenges in their adulthood. As the antioxidant defence is involved in important fitness-related traits as reproduction, survival and resistance to insecticides, our results suggest that within the scope of our study, permethrin might be beneficial but not detrimental for mosquitoes. In addition, we show that a higher antioxidant capacity is associated to an increased melanization response, and thus enhanced immunity. However, larval competition for food decreased the beneficial effects provided by the insecticidal exposure, suggesting this stimulatory effect to come along with energetical costs for the mosquitoes.

5.6 Supplementary Information for Chapter 5

Table S1. PCA model included 4 OS markers: MDA [ng/mg of abdomen] (square-root transformed), total glutathione [nmol/mg of abdomen] (log10 transformed), GSH to GSSG ratio (log10 transformed), and SOD activity [unit/mg of abdomen]. Variables were all scaled and centered for the analysis. Eigenvalues and variance explained by principal components are given. PC 1-4 are the principal components of the analysis.

	PC1	PC2	PC3	PC4
Eigenvalues	1.6555	1.0878	0.8358	0.4208
Proportion of variance explained	0.4139	0.272	0.2089	0.1052
Cumulative proportion	0.4139	0.6858	0.8948	1

Table S2. Shown are the loading values of the PCA. PC2 was chosen as an indicator of oxidative stress, as MDA (OS marker) was in opposition to total glutathione and SOD (antioxidant markers). However, the GSH to GSSG ratio (antioxidant marker) was almost not related to PC2. PC1 was not selected despite it explained a higher proportion of variance of the data, as on the PC1 axis MDA was positively correlated to glutathione or to the GSH to GSSG ratio, and was therefore not coherent as an OS indicator.

	PC1	PC2	PC3	PC4
MDA (square root)	-0.4326414	0.22662956	-0.86962127	-0.07224505
Total glutathione (log10)	-0.5684906	-0.51410901	0.09608975	0.635041
ratio GSH : GSSG (log10)	-0.6499582	-0.03087927	0.37038072	-0.66288677
SOD (log10)	0.2592183	-0.82666646	-0.31199957	-0.38998039

Chapter 6

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

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6.1 Abstract

Insecticide-treated bed-nets (ITNs) control malaria by keeping mosquitoes from reaching people sleeping under a net and by killing mosquitoes. Most tests of ITNs consider their overall epidemiological outcome without considering the different behaviors underlying their effects. Here we consider one of these behaviors: that mosquitoes can bite through the net if its user is touching it. We assayed the ability of an insecticide-sensitive strain of the mosquito Anopheles gambiae to bite through a permethrin-treated or an untreated net, and their subsequent survival and fecundity. Despite the irritancy of permethrin, 71% of the mosquitoes took blood through the ITN (vs. 99% through the untreated net). The ITN reduced the time spent biting, the blood-meal size and the fecundity, and it killed about 15% of the mosquitoes within 24 hours of feeding (vs. 5% on the untreated net). However, the mosquito's survival was much higher than what we found in WHO cone assays, suggesting that the bloodmeal increased the mosquito's resistance to the insecticide. Thus, our results suggest that the irritancy and the toxicity of ITNs are reduced when mosquitoes contact and feed on their host, which will affect our understanding of the personal and community protection offered by the ITNs.

6.2 Introduction

Insecticide-treated bed nets (ITNs) are among the most cost-effective tools used to control malaria (19, 157, 323). By reducing the number of malaria cases by 39 to 62% and child mortality by 14 to 29% (157), they help to save hundreds of thousands of people from dying of malaria every year (324). 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 (49, 325–329). Third, if mosquitoes touch the net long enough (when 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 (330, 331) 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 (332). 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 (329). Nevertheless, the insecticide reduced blood-feeding success only by a factor or about 3 (329), 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) (333); at 0.8 g/m^2 (slightly more than what was found on an Olyset net after 1 year of use (334)) 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. Community protection may, however, be maintained if mosquitoes die after they have bitten through an ITN, preventing further infectious bites. In the study mentioned above, about a third of the mosquitoes that managed to bite through the net were knocked down and died (333). This short-term effect of exposure to insecticides during biting may be complemented by long-term or sublethal effects. Thus, exposure to a Permanet 2.0 (a net treated with 0.5% deltamethrin) reduces the survival rate of mosquitoes for several days after exposure (160). Mosquitoes irritated by insecticides are likely to stop their blood-seeking behavior for several days (46)

(thus protecting the ITN-users and others from being bitten) and lay fewer eggs (46) (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 insecticide-sensitive *A. gambiae* mosquitoes to bite through a new generation of ITN and its consequences for several aspects of the mosquitoes' feeding behavior and fitness. While the efficacy of ITNs to reduce malaria prevalence is not questioned here, our goal was to understand better the properties and effects of an ITN in the scenario of mosquitoes having access to a human host by biting through the net. Also, using a sensitive strain of *A. gambiae* allowed us to establish the maximal level of protection the ITN can offer in this particular case. As a model, we used an ITN that is widely used in Africa: Olyset Plus®. This ITN is treated with 2% (w/w) permethrin and 1% piperonyl butoxide (PBO), which slows down the metabolic degradation of pyrethroids (335, 336). The presence of PBO allows this ITN to remain efficient against mosquitoes harboring metabolic resistance (328), which is particularly important in malaria endemic regions, where resistance is now wide-spread (190). We also expect that the slow degradation of the insecticide might increase the effects of the insecticide on the mosquito's survival several days after exposure.

Here we measured the proportion of mosquitoes that were able to take blood through an untreated net and through an Olyset Plus net, the time mosquitoes spent blood-feeding on the nets, and their blood-meal size. We also 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 act of blood-feeding affected the survival of the mosquitoes contacting the ITN.

6.3 Methods

6.3.1 Mosquitoes

We used the insecticide-sensitive Kisumu strain of *Anopheles gambiae s.s.* originating from western Kenya (196). We confirmed the sensitivity of our colony by exposing 100 mosquitoes to 0.75% permethrin (WHO filter papers (337)) for one hour and finding that all mosquitoes died within 24 hours. Throughout our experiment, the 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.

6.3.2 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 gave them the opportunity to blood-feed for 8 min on Gaël Hauser's (GH) 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.

Blood meal size

As previously described by Briegel and coworkers (338), 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 porcine haematin (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 replicated samples).

Body size

Wing length was used as a proxy for the mosquito's body size. We placed the wings onto a slide, took a digital photograph of each wing and measured it with the software ImageJ v1.51 (198) from the distal end of the alula to the tip of the wing (the end of the vein R3) without the fringes.

6.3.3 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 (339). 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 GH's arm. Directly after the blood meal, we measured the resistance of these mosquitoes and of approximatively 250 unfed females with the WHO cone bioassay (340). 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.

6.3.4 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 (263)). 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) (200) and with the Rstudio interface (341) (version 1.1.456). Graphs were made using R, and edited (labels, colors and format) using Inkscape (version 0.92.2).

6.3.5 Effect of ITN on blood-feeding behavior

Blood-meal size

We described the blood-meal in five ways: 1) the proportion of mosquitoes that tried to bite at least once (*biting success*), 2) the proportion that succeeded to take blood during the 8 minutes they were allowed to (*feeding success*), 3) the time required for the mosquitoes to start biting through the net (*time to bite*), 4) the time they spent feeding until they detached or until the end of the allocated time (*feeding time*), and 5) 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) were 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 (204))), where the mosquitoes that did not try to bite were censored. 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. 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 mosquitoes 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 GLM with binomial error distribution. Longevity was analyzed with a Cox proportional hazards regression model. Both analyses included the type of net as an explanatory factor.

6.3.6 Effect of bloodmeal on resistance

We built time-response models with the *drm* function (*drc* library in R (342)). We used a 2-parameter 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.

6.4 Results

6.4.1 Effect of ITN on blood-feeding behavior

Blood meal

101 mosquitoes were tested on an untreated bed net and 85 on an Olyset Plus net. All of the 101 mosquitoes tested on an untreated net 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 obtained some blood (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). If mosquitoes tried to bite, it took them an average of 35.0 seconds (± 12.9 (95% CI)) to start biting through the untreated net, but about 50% 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 stopped feeding after 175 sec and none of them fed for more than 300 seconds (**Fig. 1a**; X^2=198.35, df=1, p<0.001).

Feeding time was 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).

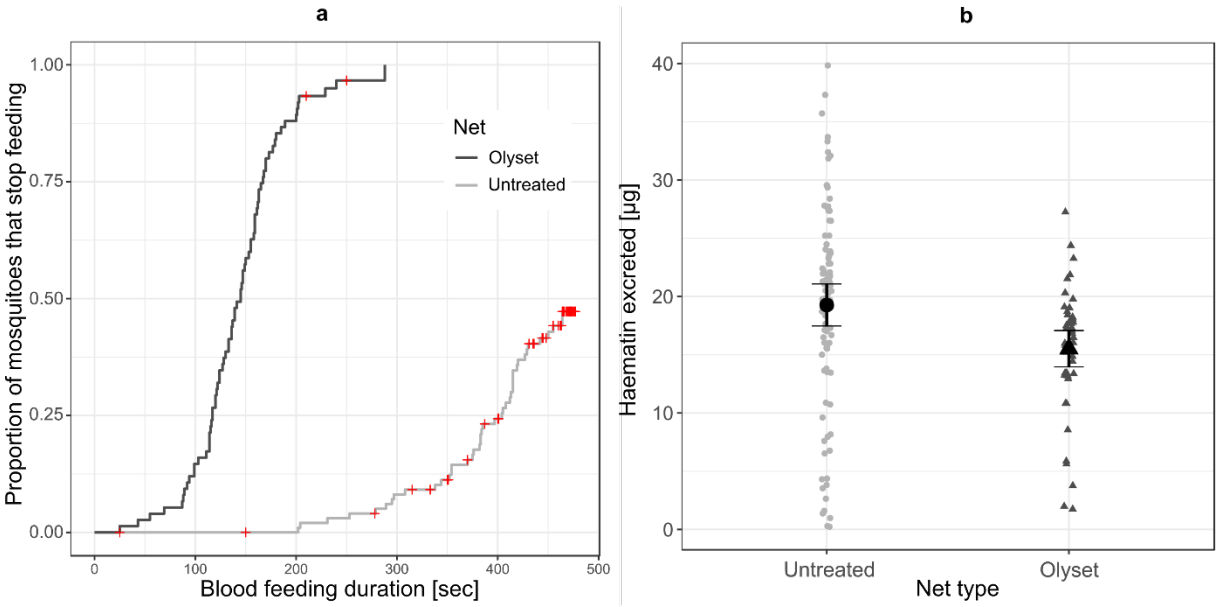

Figure 1. Blood feeding duration and subsequent haematin excretion. (a) Cumulative proportion of mosquitoes with a given duration of blood-feeding (time between the beginning of probing and detaching). Red crosses represent the mosquitoes that did not spontaneously detach and were thus still biting at the end of the allocated time. (Untreated: N = 101, Olyset: N = 75). (b) Quantity of haematin excreted by mosquitoes in function of the type of net they were able to feed through (Untreated: N = 92, Olyset: N = 46). Error bars show the 95% confidence intervals.

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). The number of eggs increased with the mosquito's wing length ($F_{1,96}$=9.78, p=0.002).

In a second model, haematin level was included in the multiple regression to look at possible mechanisms responsible for the observed difference. Haematin excretion level was positively correlated with eggs number (**Fig. 2b**; $F_{1,91}$=36.56, p<0.001), and that the type of net no longer had a statistically significant effect ($F_{1,91}$=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.

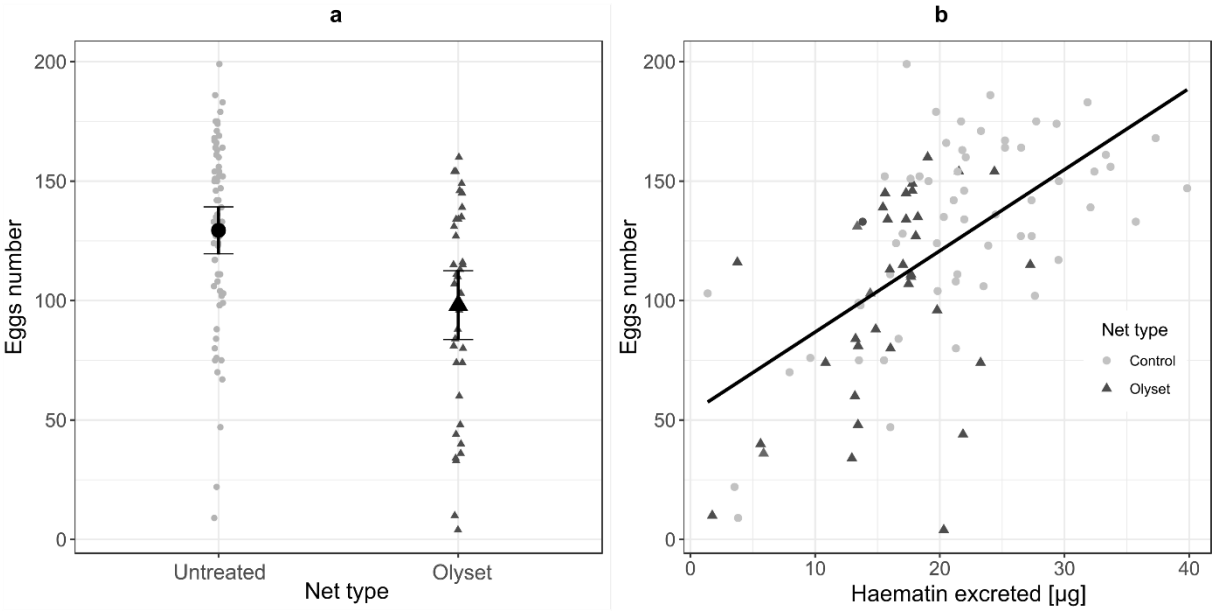

Figure 2. Fecundity and its correlation with haematin level. (a) Quantity of eggs laid by mosquitoes in function of the type of net they bit through (Untreated: N = 63, Olyset: N = 37). Error bars show the 95% confidence intervals. (b) Relationship between the number of eggs laid by mosquitoes and the quantity of haematin they excreted. Black triangles represent the mosquitoes that fed through the Olyset plus net, and the grey dots represent those that fed through the untreated net (Untreated: N = 59, Olyset: N = 36). The line shows the linear regression.

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, half of the mosquitoes died within 6 days, whether they had fed through the ITN or through the untreated net (**Fig. 3;** X^2=1.79, df=1, p=0.18).

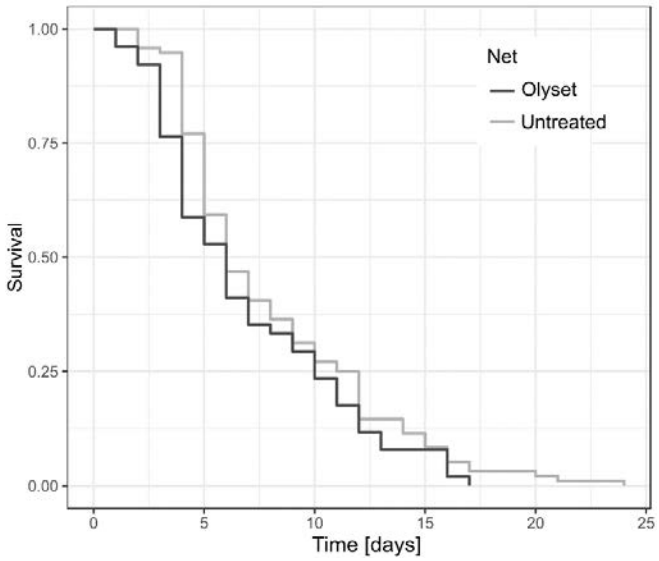

Figure 3. Mosquito's longevity in function of the type of net they could feed through. Survival is shown starting from 24 h following the blood meal (day 1). The black line represents the survival of mosquitoes that bit through the Olyset net; the grey one represents the mosquitoes that fed through the untreated net (Untreated: N = 96, Olyset: N = 51).

6.4.1 Effect of bloodmeal on resistance

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

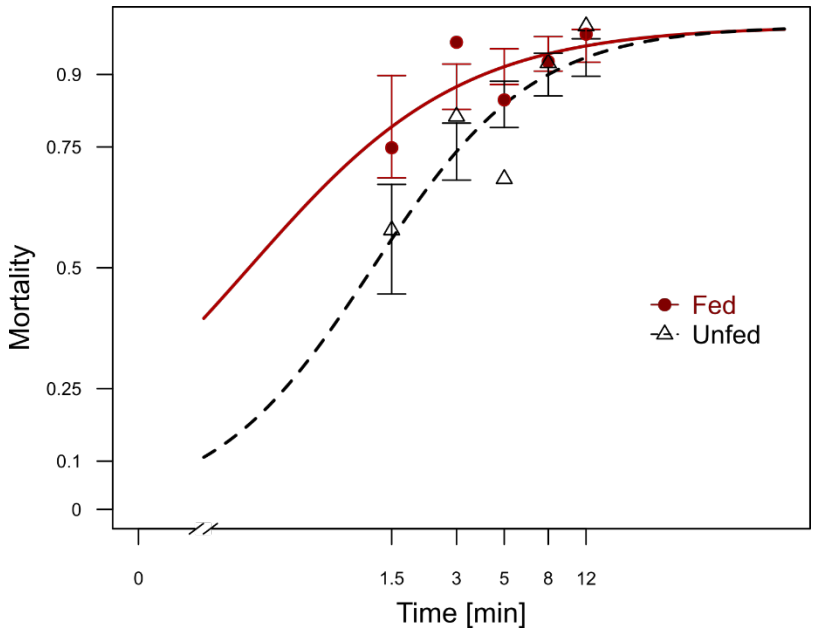

Figure 4. Time–mortality curve of unfed (black dashed line) and freshly blood fed (red solid line) females exposed to an Olyset Plus net following the WHO cone bioassay procedure. Points represent averages mortality at each time, five exposure durations were assessed for both unfed and fed mosquitoes. Error bars show the 95% confidence intervals of the regression lines.

6.5 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 (42, 325, 332). However, despite that irritancy, 88% of the mosquitoes tried to bite and 71% succeeded to take a blood meal through the net. This feeding through the permethrin-treated net came with fitness costs for the mosquitoes, for it reduced their chance to survive and their fecundity, corroborating the results from previous studies (46, 160, 333).

The irritancy of the Olyset plus net was confirmed by the fact that the mosquitoes took more time to start blood feeding and spent less time feeding through the treated net than through 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 (42, 325, 332). 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 sublethal

exposure to pyrethroids may alter the number of eggs mosquitoes are able to produce (46). Our results, however, suggest that this reduction of fecundity after an ITN exposure is not a direct effect of the toxicity of the insecticide, but rather caused by a reduced feeding time. Overall, our results suggest that the ITN may not only prevent mosquitoes from biting by irritating them, but may also reduce the density of mosquitoes through a reduction of their fecundity.

Although the Olyset Plus net strongly irritated the mosquitoes, it prevented only 12% of the mosquitoes from trying to bite through the net, and 80% of the ones that tried to bite were able to take blood. Therefore, when the user of net touches the net, the personal protection offered by it may be reduced, which would have strong implications for the epidemiology of mosquito-borne diseases like malaria, if our results were confirmed in the field. Indeed, since *Plasmodium* sporozoites (the stage that is infectious for human) are mostly released at the beginning of the bite, the large proportion of mosquitoes that bite, even if only for a short time, will keep the transmission potential high (343, 344). It is, however, worth noting that our study focused on the ability of mosquitoes to bite through an Olyset Plus net, and did not consider other factors that may reduce the number of mosquitoes reaching the net in field condition, like long range repellency.

Since Olyset Plus is treated with 1% piperonyl butoxide (PBO), which slows down the metabolic degradation of pyrethroids (335, 336), we expected to see an effect of exposure to the insecticide on survival throughout the mosquito's life. However, 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. Thus, despite PBO, permethrin appeared to have only a short-term impact on survival.

Because the WHO recommend that ITNs kill 80% of the mosquitoes within 24h after being exposed for a duration of 3 min in a cone assay (340), we expected that most of the mosquitoes would not survive after a blood meal through the Olyset Plus net. However, we found a surprisingly low impact on survival: only 15% 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 in the blood feeding experiment– should have experienced 68% (unfed females) or 85% (fed females) mortality at 24h. A possible explanation for the high survival is that, contrarily to the mosquitoes used in the cone bioassay, which were all exposed in a similar way, the mosquitoes used in the feeding experiment had the choice to bite (and being exposed) and bit for a variable amount of time. 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 assume that all the mosquitoes that did not bite would have bitten and then died within 24h, the recorded mortality would only have been increased from 15% to 39%, which is still considerably lower than what was predicted by the cone assay 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 presence of blood alone 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 sensitive than unfed mosquitoes in the cone assay. Therefore, we briefly introduce two possible mechanisms that might be implied. First, both a blood meal (150) and pyrethroid exposure (155, 210) increase the concentration of reactive oxygen species (ROS), which is quickly followed by a higher expression of different antioxidants in the midgut and the fat body (150), as found in some pyrethroid-resistant mosquito strains (220). Thus, one possibility may involve an oxidative based interplay between the blood meal and permethrin exposure, which may reduce the damage induced by the insecticide. However, the fact that this mechanism would no longer be active a few minutes after the blood meal, as we tested in the cone assay, would be puzzling. A second possibility may involve temperature. During a blood meal the body temperature of mosquitoes increases rapidly (345). Because the toxicity of pyrethroids decreases at higher temperatures (188, 346, 347), blood-feeding mosquitoes might suffer less from being exposed to pyrethroids. This hypothesis is also more consistent with the results obtained via the cone bioassay: fed mosquitoes had already cooled down when they were put on the Olyset Plus net and therefore were no longer protected by the high temperature reached during the blood meal.

The possibility for the blood meal to mitigate toxicity of pyrethroids when mosquitoes are biting through the net may be of high importance when evaluating ITNs efficiency and their insecticidal properties. Indeed, our results suggest that the protection they offer might be impeded in that particular case. Although we confirmed that the commercially available permethrin-treated Olyset Plus net was irritant, 88% of the tested mosquitoes tried to bite through the net and most of them succeeded to take blood and further survive. It follows that mosquitoes that have the possibility to bite through an Olyset Plus net may potentially acquire one or more parasites and transmit them, affecting both the personal and community protection that the net confers in standard experimental conditions (328).

To conclude, while the efficiency of Olyset Plus net to reduce malaria prevalence has already been demonstrated, our results showed that the insecticide itself only slightly prevents mosquitoes from biting through the net when they are given the opportunity. The time mosquitoes spent feeding on the net was reduced due to its irritant property, which reduced mosquito fecundity. Finally, taking a blood meal helped mosquitoes to survive their exposure to permethrin through a physiological mechanism that remains to be determined. Altogether, our results point out the importance to avoid skin contact with the net to guarantee a maximal protection for both the user and the community.

Chapter 7

Insecticide at sublethal concentration hastens the evolution of resistance of mosquitoes selected on a PBO-permethrin treated net

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7.1 Abstract

Because of the wide use of insecticides in malaria endemic areas, resistance in mosquito vectors has rapidly widespread. To prevent this resistance from jeopardizing the efficacy of vector control tools, a new generation of insecticide-treated bed nets (ITNs) now includes a synergist, PBO, that artificially restores mosquito's susceptibility to insecticides. Consequently, PBO-ITNs maintain a selection pressure on both insecticide-sensitive and resistant mosquitoes. However, whether the presence of the synergist prevents or contributes to the evolution of resistance, and how this resistance may eventually result in a decreased efficacy of these nets, is poorly understood. Here, we tested these ideas using the malaria vector *Anopheles gambiae* and the PBO-ITN Olyset Plus® net. We selected mosquitoes by feeding them through an untreated or an Olyset Plus® net for 11 generations, and because in nature mosquitoes are likely to be exposed to insecticide residues at larval stage, half of the mosquitoes were additionally reared with a sublethal concentration of permethrin (pyrethroid). After the selection phase, mosquitoes were tested for their resistance to permethrin and evaluated for their feeding behavior, fecundity, and longevity when given the possibility to feed through an Olyset Plus® net. We found that insecticide residues may act as a catalyzer for the evolution of resistance, as feeding through an Olyset Plus® net only selected for a higher resistance in mosquitoes that were additionally exposed to a sublethal dose of permethrin at larval stage. Our results also showed that moderate resistance may already increase mosquitoes' biting success and survival when given the possibility to bite through an Olyset Plus® net, but that mosquitoes still suffered from significant fitness costs.

7.2 Introduction

To reduce the burden of malaria, the use of insecticide-treated bed nets (ITNs) is, along with indoor residual spraying (IRS), at the core of the vector control strategy of the World Health Organization (348). In particular, ITNs have been shown to be one of the most powerful tool to prevent malaria transmission (19, 157) and 68% of the cases averted since 2000 have been attributed to its use (349). However, despite the proportion of the population protected with ITNs has continuously increased during the 2010-2017 period, malaria incidence in the population at risk as well as global malaria prevalence have remained globally stable since 2015 (1). To explain this discrepancy, the rapid evolution of insecticide resistance in the mosquito vector has been suspected to jeopardize the efficacy of vector control strategies (111, 112). In particular, a high insecticide resistance may decrease the contact irritancy of ITNs (42, 350) as well as their insecticidal property (351). A recent study additionally showed that when biting through an ITN, survival and fecundity of resistant mosquitoes were not affected (161), contrarily to what was found with insecticide sensitive mosquitoes (299).

To tackle the resistance issue, a new generation of ITNs nets that include a synergist have been developed. These ITNs (e.g. Olyset Plus® or Permanet 3.0) include piperonyl butoxide (PBO), which is a synergist that inhibits mosquito's detoxifying enzymes (335, 352) leading to a full (353) or partial (354) restoration of mosquito sensitivity to insecticides. Compared to standard ITNs, PBO-ITNs were shown to be more effective in killing insecticide resistant mosquitoes and in decreasing their blood-feeding success (355). By doing so, PBO-ITNs are also expected to impose a selection pressure on mosquitoes that may lead to an increase of resistance. A few cases of selection for resistance have been reported (166–168) following the distribution of standard ITNs. However, to which extent a PBO-based ITN can contribute to select for resistance is still unknown.

Another important driver of resistance is agriculture (162, 165, 356). Indeed, before mosquitoes may contact ITNs, they are first likely to be exposed to various chemicals at larval stage: fresh water and sediments of urban and agricultural areas being polluted at global scales by various insecticides (28) including pyrethroids (182, 183). Therefore, in malaria endemic areas, both mosquito stages are likely to be exposed to insecticides. Surprisingly, the interaction between both types of stressor and their consequences for the evolution of resistance have received little attention.

In addition, if the efficacy of PBO-ITNs against resistant mosquitoes has already been demonstrated in standardized assays (mainly assessing short-term mortality and blood-feeding success) (328, 354), it may be worth assessing the effect of those nets on additional mosquito traits relevant for malaria transmission to better evaluate of the consequences of insecticide resistance (117, 357).

The aim of our study was twofold: first, to test how both insecticide residues in water and a PBO-ITN contribute to the evolution of resistance, and second, to assess the consequences of such selected resistance on several aspects of mosquito's blood feeding success and fitness when given the possibility to bite through the insecticidal net. During the experiment, we selected a sensitive strain of the malaria vector *Anopheles gambiae* during 11 generation by feeding mosquito females through an the PBO-ITN Olyset Plus®. This ITN is widely used in malaria endemic areas and is treated with 2% (w/w) permethrin (a pyrethroid) and 1% PBO. In a former experiment, we showed that mosquitoes feeding through that net suffer from fitness costs in terms of survival and fecundity (299). To force mosquitoes to take a blood-meal through the net is thus expected to impose a significant selection pressure. In addition, mosquito larvae were reared either in water or with a sublethal dose of permethrin to simulate a polluted environment. Both larval treatment and adult selection were combined in a full factorial design.

After 11 generations, the level of resistance of all four treatments was assayed, and mosquitoes were tested for their ability to feed through the Olyset Plus® net by measuring several traits related to their feeding behavior, as well as their subsequent mortality, fecundity and longevity. This allowed us to assess several overlooked aspects of the protection provided by the ITN against partially resistant mosquitoes.

7.3 Material and Methods

7.3.1 Mosquitoes

The experiment was done with the insecticide-sensitive Kisumu strain of *Anopheles gambiae s.s.* originating from western Kenya (166). We confirmed the sensitivity of our colony by exposing 115 mosquitoes in 6 replicates to 0.75% permethrin for one hour following the WHO test procedure (199). All mosquitoes died within 24 hours. Throughout our experiment, the mosquitoes were kept in an insectary maintained at 26.5 ± 0.5 C° and 75 ± 5 % humidity with a 12:12 hours dark:light cycle.

7.3.2 Determination of the sublethal dose of permethrin

Four successive dose-mortality trials were done with permethrin concentrations varying from 0.001 to 0.3 µg/L. Permethrin solutions were made from a 0.1 µg/mL stock solution of solid permethrin (Sigma-Aldrich Inc., St. Louis, Missouri) dissolved in pure ethanol. Freshly hatched *Anopheles gambiae* larvae were reared by groups of 200 in 35 x 25 x 7 cm glass trays containing 800 mL of 0.001 % to 0.3 % ethanol with the desired concentration of permethrin. For the control solution, the concentration of ethanol was fixed at 0.1% (volume per volume) (789 µg/L). This concentration was not found to induce significant mortality in any of the trials (tested against a mortality of 0/200 using 2-sample tests for equality of proportions (*prop.test* function in R). Mortality was recorded at the end of the development

period by counting the pupae. Based on the results, we used a concentration of 0.02 µg/L in the selection experiment. 2-sample tests for equality of proportions confirmed that larval mortality recorded at this concentration was not different of what was observed in control trays. The concentration of ethanol used for the experiment was fixed at 0.02% v/v (157.8 µg/L).

7.3.3 Selection

Mosquitoes were selected for their ability to bite through an Olyset Plus® net during 11 generations, with larvae being additionally exposed or not exposed to permethrin. To start the selection, freshly hatched mosquito larvae from the colony were haphazardly divided into the four treatments: larvae were reared either in a permethrin solution (0.02% ethanol supplemented with permethrin (final concentration of 0.02 µg/L)) or in a control solution (0.02% ethanol) (hereafter: *larval selection treatment*), and adults were blood fed either through an Olyset Plus® net or an untreated net in a full factorial design (hereafter: *adult selection treatment*). It is worth noting that the name *larval selection treatment* refers to the phase of the experiment in which this treatment is applied (selection phase) and not the dose of the insecticide, as it was not designed to impose a direct selection pressure (the dose of permethrin being sublethal).

Rearing of larvae

Each of the four treatment combinations was replicated three times, thus a total of 12 selection groups (hereafter: *lines*). The treatment in which larvae were reared in control solution and adults fed through an untreated net was the selection control. At each generation and for each selection line, 400 larvae were divided into two glass trays (200 larvae per tray) and provided daily with Tetramin Baby® fish food according to their age, with: 0.04, 0.06, 0.08, 0.16, 0.32, 0.32 and 0.6 mg/larva for day 0,1,2,3,4,5 and 6+ respectively (adapted from (196)). Pupae were collected every day until all larvae pupated and transferred in 21 x 21 x 21 cm plastic cages (1 cage per line). Adults had constant access to a 6% sucrose solution. During the 10^{th} generation of selection, pupae were precisely counted at the end of the development (in 1 of the 2 trays per line) to assess the effect of the treatments on larval mortality during the selection process.

Blood feeding

Adults were blood fed 15 days after eggs hatched, that is at age 4 to 7 days. The upper part of each cage had a 15 cm diameter hole covered with a removable untreated net. This net was replaced by either a piece of Olyset Plus® net or a piece of an untreated net of similar mesh size (Pharmavoyage ® Trek) according to the selection treatment, and mosquitoes were given the possibility to blood feed through the net on Gaël Hauser's (GH) arm for 8 minutes. At the end of the blood meal, nets were removed to ensure that permethrin exposure only affected mosquitoes during the 8 minutes they were allowed to take blood. Mosquitoes were then kept in the cages for 2 to 4 hours before blood-fed

females were transferred in new cages. They were given the possibility to lay eggs after 48h, and eggs were collected the following day. Finally, the resulting larvae were transferred in trays to form the next generation. An entire cycle took 18 days.

7.3.4 Final experiment

Traits measured

We measured the effects of our selection treatments on the evolution of resistance to permethrin, the capacity of mosquito females to feed through an Olyset Plus® net and the consequences of this blood meal on their survival and fecundity.

General design

After the 11th generation, a "blank" generation (no larval or adult treatment was applied) was reared. The final tests were performed on the offspring of that blank generation to ensure that the observed effects would be the consequences of evolution and not the results of maternal effects. Final experiment was run in two experimental blocks, the mosquitoes of both blocks coming from the same parental (blank) generation. The adults of the blank generation were therefore blood fed twice: first when they were 2-4 days old, and second when they were 13-15 days old. In total, 14'400 larvae divided in 72 trays (3 trays of 200 larvae per line per block) were reared, resulting in 72 cages. Of these, 48 cages were used for the resistance assay (ca. 400 females per line), and 24 cages were used for the feeding assay (ca. 200 females per line). Both the resistance and the feeding assays were performed in each of the two blocks.

Resistance assay

Resistance was assayed by exposing 2 to 4 days old females to 0.75% permethrin impregnated paper in WHO test tubes (199). Five different exposure durations were chosen to obtain a mortality ranging between 10% and 75%: 4, 8, 14, 22 and 32 minutes. Four replicates of 20 females per line per exposure duration were assayed (2 replicates per block). After the exposure, females were transferred to 120 mL plastic cups closed with a net, and a cotton ball soaked in a 6% sucrose solution was deposited on the net. Mortality was recorded after 24 hours, and mosquitoes that lost all their legs or could not properly stand or land were considered dead. We also tested for any potential mortality induced by the manipulation itself by exposing mosquitoes to WHO pyrethroid-control papers (impregnated with mineral oil). To do that, one replicate of 16-22 females per line was exposed during 32 minutes to the control paper. No mosquito died within 24 hours, confirming that the manipulation itself did not induce mortality, irrespective of the selection treatment.

Feeding assay, lay and survival

The ability of mosquitoes to feed through a permethrin impregnated bed net (Olyset Plus®) was assessed by following the methods described in Hauser et al. (299) with a few modifications. Mosquito females were transferred individually in 120 mL plastic cups covered either with a piece of Olyset Plus® net or an untreated (Pharmavoyage® Trek) net and were given the possibility to bite on GH's arm through the net for 8 minutes. Right after the blood meal, females were visually assessed to determine whether or not they took blood, unfed females were removed and fed females were transferred in a new 120 mL plastic cups closed with a piece of untreated net. A cotton soaked in a 6% sucrose solution was put on the net. After 48 hours, females were moved in new cups containing a basket shaped filter paper and water in the bottom for them to lay their eggs. The filter papers containing the eggs were removed after 4 days and a picture of each batch of eggs was taken to facilitate the counting. Mosquitoes were finally kept in plastic cups and mortality was checked daily until all mosquitoes died.

7.3.5 Statistical analyzes

All analyzes and graphs were done using the R software (version 3.6.1) (200) and with the Rstudio interface (341) (version 1.2.1335). Significance of the variables were tested using the Anova function of the *car* library (201). We used a type III anova in the case of a significant interaction, and a type II anova otherwise. Non-significant interactions were removed from final models.

When relevant, post-hoc analyzes were performed with *emmeans* (using Estimated Marginal Means (EMM)) and *pairs* functions of the *emmeans* library in R, with p-values being adjusted using the *mvt* method. 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 (263). For Cox models, the assumption of proportional hazards was tested with the *cox.zph* function from the *survival* library (204).

Larval mortality during selection

Larval mortality of each line during the 10th generation was tested using a Generalized Linear Mixed-Effect Models (GLMM; *glmer* function from the *lme4* (358) library in R) with binomial error distribution. The survival recorded in each tray (i.e. line) was included as the response variable, and larval selection and adult selection treatments were included as explanatory variables. The line was included as a random factor. The model was weighted by the total number of larvae initially counted in each tray.

Resistance to permethrin

To test for differences in resistance between the treatments, we used a GLMM with binomial error distribution, where the mortality recorded in each test tube was the response variable, and the model was weighted by the number of individuals in each tube. Larval exposure during selection (larval

selection treatment) and the net they were selected on (adult selection treatment) where included as explanatory factors, time of exposure and block were set as covariates, and the lines and replicates (within lines) were set as random factors. LT50 values (the time of exposure leading half of the mosquitoes to die) were also estimated for each treatment using the *ED* function of the *drc* library in R (342). We used a 2-parameters log-logistic model (*drm* function of the *drc* library), setting lower and higher mortality to 0 and 1, with the mortality of each test tube as the response variable and the treatment as explanatory variable. The model was weighted by number of mosquitoes per test tube. This model was only used to compute LT50 values and not to test hypotheses, as it does not allow to include covariates or random components.

Blood feeding behavior

The behavior of females that had the possibility to bite through an untreated or Olyset Plus® net for 8 minutes was measured in four ways: 1) the proportion of mosquitoes that tried to bite at least once (*biting success*), 2) the time required for them to start biting (*time to bite*), 3) the proportion of mosquitoes that succeeded to take blood (*feeding success*), and 4) the time they spent feeding until they detached or until the end of the allocated time (*feeding time*).

Biting success and feeding success were analyzed using a GLMM with binomial error distribution. Time to bite and feeding time were analyzed with a mixed-effect Cox proportional hazard regression model (from *coxme* (359) library in R) . For the analysis of time to bite, mosquitoes that did not try to bite where censored. For the analysis of feeding time, mosquitoes that were still feeding at the end of the allocated feeding period were censored. In all four models, larval selection treatment, adult selection treatment and the type of net mosquitoes had to feed through were included as explanatory factors, the experimental block was added as a covariate, and the line was included as a random factor.

Fecundity and survival

Fecundity was analyzed as both the proportion of mosquito females that laid at least one egg (*laying success*), and the number of eggs laid by each of these females. Laying success was analyzed with a GLMM with binomial error distribution. The number of eggs laid was analyzed with a Linear Mixed-Effect Model (LMM).

Survival was analyzed as both the proportion of mosquitoes that survived the first 24 hours following the blood meal (*24h survival*), and the longevity of mosquitoes after the first 24 hours. The survival rate after 24 hours was analyzed with a GLMM with binomial error distribution. Longevity was analyzed with a mixed-effect Cox proportional hazard regression models.

These models included larval selection treatment, adult selection treatment and the net type were as explanatory variables, the experimental block was added as a covariate, and the line was included as a random factor.

Correlation between mosquito traits and resistance

In addition, we tested whether the level of resistance of mosquitoes was associated with their performance during the final tests. As resistance level may differ between the lines but also from one experimental block to the other, we considered each line during each experimental block as a statistical unit. For each of these units, we estimated the resistance level by calculating the LT50 value using the method described above, and then calculated the corresponding performance on both the untreated and the Olyset Plus® net during the final tests (i.e. biting success, time to bite, feeding success, feeding time, laying success, egg number, 24h survival, and longevity). Finally, we tested the effect of resistance on each of these traits using LMMs, with the LT50 value and net type as explanatory variables, the block as a cofactor, and the line inside the block as a random factor. To reach normality of residuals, feeding time was square root transformed, feeding success and biting success were log transformed, survival was cubed transformed, and longevity was squared. No transformation was required for the other variables.

7.4 Results

7.4.1 Larval mortality during selection

The survival of the larvae of the 10th generation was lower by 2% in lines that were fed on an Olyset Plus® net (97.4 % (95 % CI: from 96.5 to 98.2 %) against 99.4 % (95 % CI: from 98.8 to 99.7 %); χ^2=8.23, df=1, p=0.004). However, there was no effect of larval exposure (χ^2=1.13, df=1, p=0.29) and no interaction between both treatments (χ^2=2.37, df=1, p=0.12).

7.4.2 Resistance

After 11 generations, we found that larval selection or adult selection treatments alone did not affect mosquitoes' resistance level (χ^2=0.56, df=1, p=0.45; and χ^2=1.71, df=1, p=0.19 respectively), but that both treatment interacted significantly (χ^2=14.82, df=1, p<0.001). Pairwise comparisons showed that mosquitoes that were applied both selection treatments (reared in permethrin and fed through an Olyset Plus® net) were more resistant than the other treatment combinations (all p<0.001, **Fig. 1**), but that there was no difference between the other treatments (p>0.17). LT50 in the most resistant treatment was 42% higher than in unselected control (**Table 1**).

Table 1. Estimations of the Lethal Time 50 (the time of exposure to permethrin inducing a mortality of 50 %) with the 95 % confidence interval for all 4 selection treatments: *C-Untreated* (larvae reared in a control solution, adult fed through an untreated net), *P-Untreated* (larvae reared in permethrin (0.02 µg/L), adults fed through an untreated net), *C-Olyset* (larvae reared in a control solution, adults fed through an Olyset Plus® net), and *P-Olyset* (larvae reared in permethrin, adults fed through an Olyset Plus® net).

Selection treatment	LT50	95 % CI
C-Untreated	16 min 36 s	54 s
P-Untreated	15 min 38 s	56 s
C-Olyset	17 min 56 s	18 s
P-Olyset	23 min 25 s	24 s

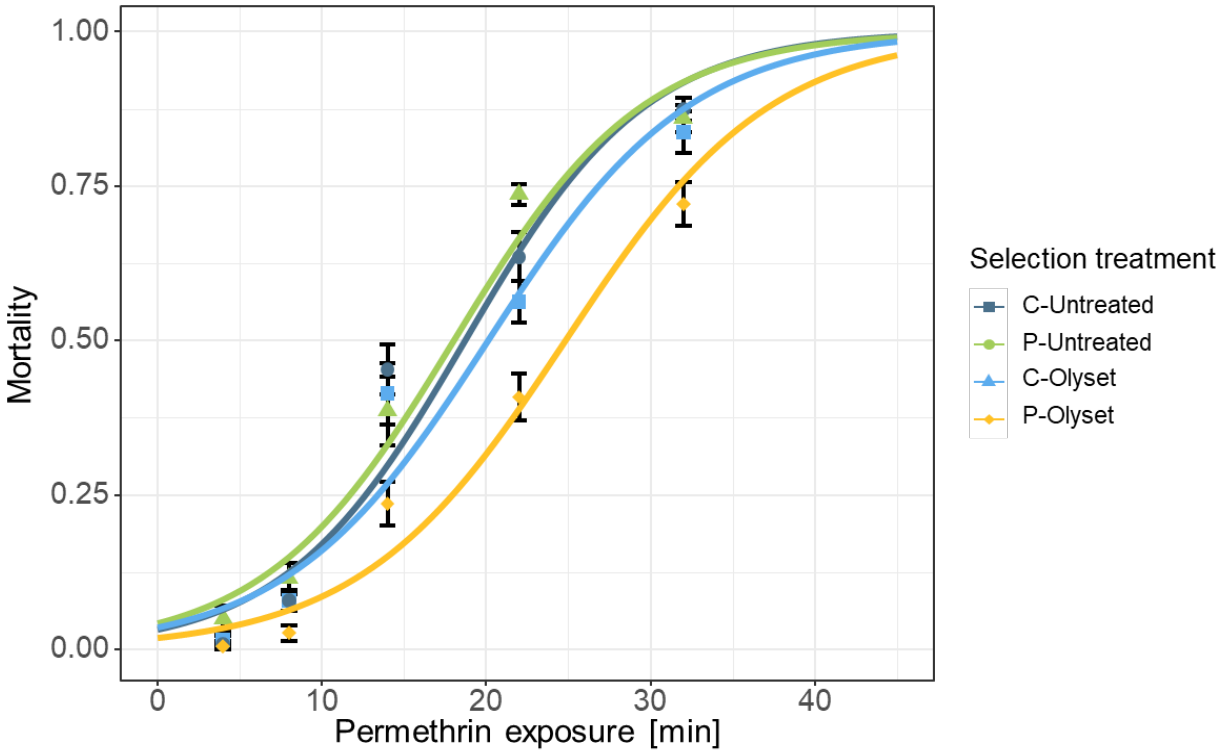

Figure 1. Dose (time) – mortality curve for the four selection treatments (treatment abbreviations are described in the legend of Table 1). Error bars show the 95% confidence intervals of the means based on test replicates (N=12 (4 replicates per line, 3 lines per treatment)).

7.4.3 Feeding behavior

Biting success

414 mosquitoes were tested on an untreated net and 957 on an Olyset Plus® net. 403 out of the 414 tried to bite through the untreated net (97.3% (95% CI: from 95.3 to 98.5%)) whereas 688 out of the 957 tried to bite through the Olyset Plus® net (71.9% (95% CI: from 69.7 to 74.6%); χ^2=71.44, df=1, p<0.001; **Fig. 2**). We also found a positive effect of both larval selection (χ^2=6.99, df=1, p=0.008) and adult selection (χ^2=4.07, df=1, p=0.04) on biting success, and a significant interaction between both treatments (χ^2=7.38, df=1, p=0.006, **Fig. 2**). Other interactions were not significant (all p>0.45). A contrast analysis showed that adult selection positively increased biting success of mosquitoes that

were also reared in permethrin (z ratio=2.02, p=0.044) but tended to decrease that of mosquitoes that were reared in a control solution during the selection phase (z ratio=-1.82, p=0.069).

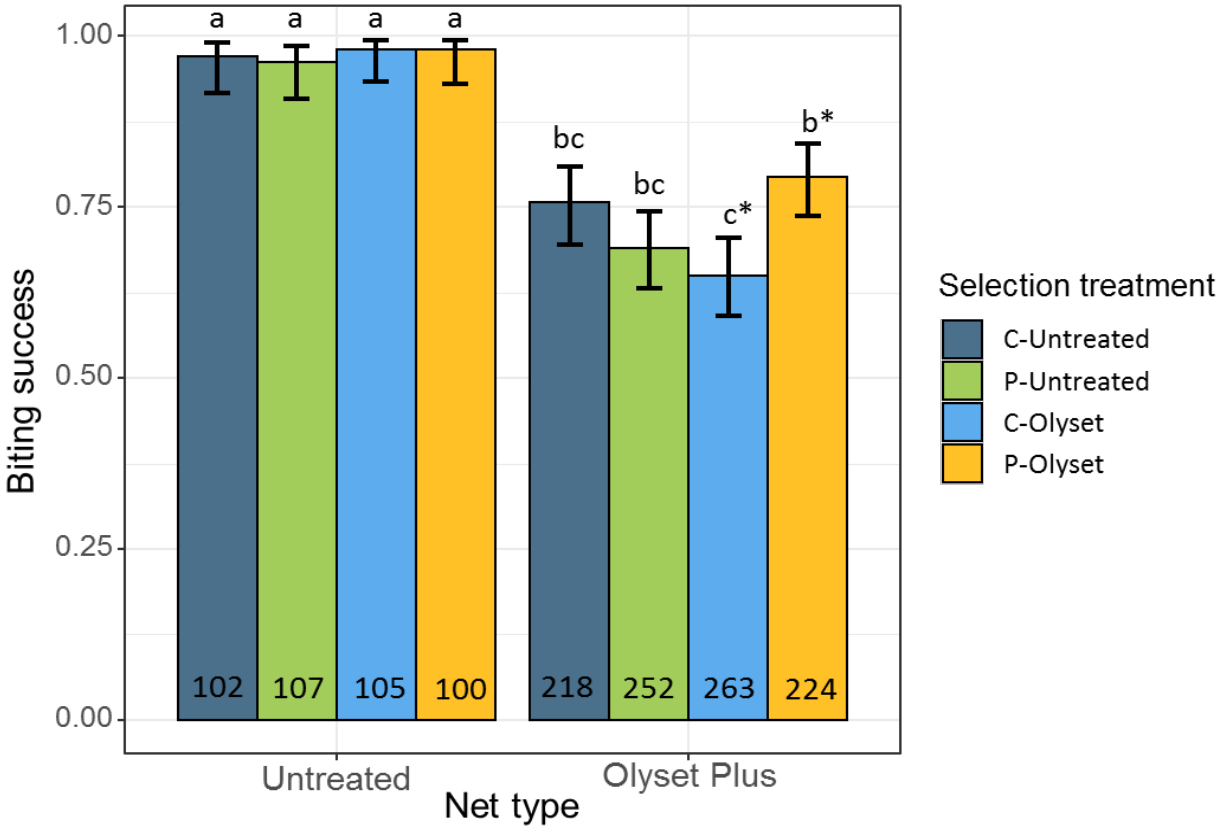

Figure 2. Proportion of mosquitoes that bit through the untreated or Olyset Plus® net according to their selection treatment (treatment abbreviations are described in the legend of Table 1). Error bars show the 95% confidence intervals. *Different letters indicate significant differences based on pairwise comparisons, except for the difference between b* and c* (non-significant trend (p=0.09)). The number of individuals of each treatment is given in the bottom of each bar.

Time to bite

Mosquitoes that had access to GH's arm through an Olyset Plus® net took 53% more time to bite than those that bit through an untreated net (83.0 ± 6.3 s (95% CI) against 54.2 ± 6.3 s; χ^2=49.85, df=1, p<0.001). There was no effect of larval selection or adult selection treatments on the time mosquitoes took before biting (χ^2=0.45, df=1, p=0.51; and χ^2=0.95, df=1, p=0.33 respectively), and no interaction was significant (all p>0.29).

Feeding success

Feeding success was reduced by 43% when the blood meal was taken through an Olyset Plus® net compared to an untreated net (54.4% (95% CI: from 51.3 to 57.6%) against 96.1% (95% CI: from 93.8 to 97.6%); χ^2=132.46, df=1, p<0.001). Feeding success was not affected by larval selection (χ^2=0.02, df=1, p=0.89) or adult selection treatments (χ^2=0.10, df=1, p=0.75) or their interaction (χ^2=2.09, df=1, p=0.15), and no other interaction was significant (all p>0.36).

Feeding time

Nearly 75 % of the mosquitoes that bit through an untreated net were still feeding after 300 s, while more than 75 % of the mosquitoes feeding through an Olyset Plus® net had already left after 200 s (χ^2=702.7, df=1, p<0.001; **Fig. 3**). However, we found no effect of larval selection (χ^2=2.54, df=1, p=0.11), adult selection (χ^2=0.19, df=1, p=0.66) or of any tested interaction (all p>0.36; **Fig. 3**).

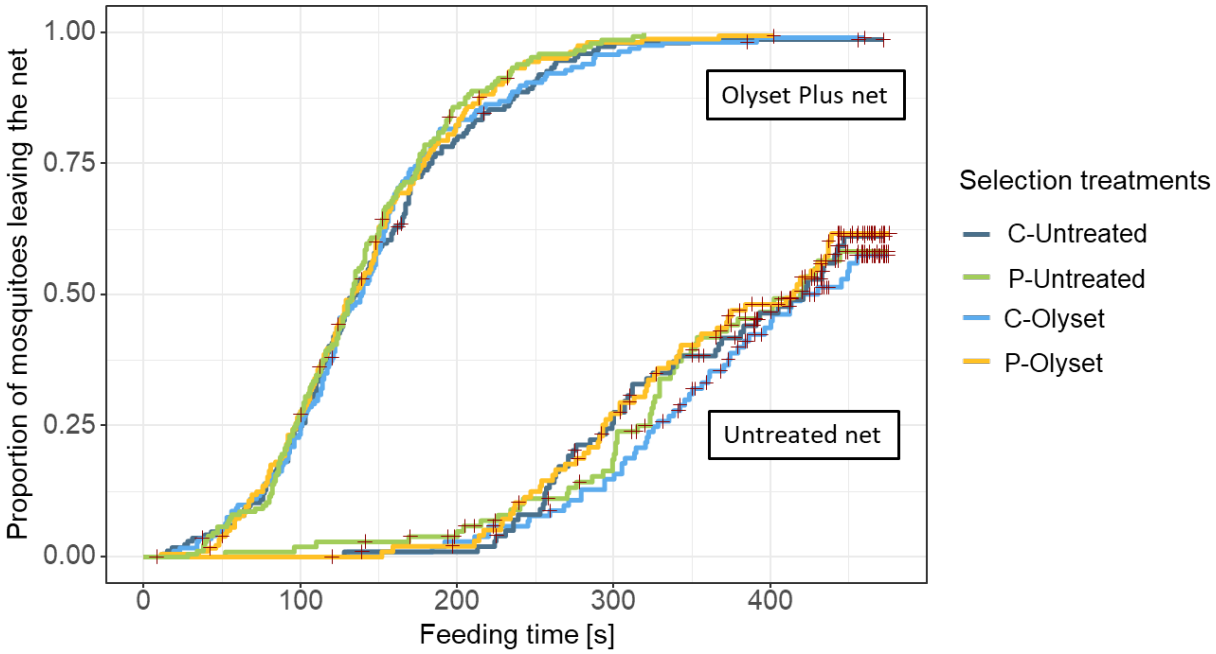

Figure 3. Blood feeding duration. Lines represent the cumulative proportion of mosquitoes leaving the net from the moment they start probing to the moment they detach. Red crosses represent the mosquitoes that were still biting at the end of the allocated feeding period. On Olyset Plus® net, N=165, 174, 171 and 178, and N=99, 103, 103 and 98 on untreated net for treatments *C-Untreated, P-Untreated, C-Olyset,* and *P-Olyset* (treatment abbreviations described in the legend of Table 1).

7.4.4 Fecundity and survival

Altogether, 692 out of 735 (94.1% (95% CI: from 92.2 to 95.6%) mosquito females laid one egg or more, with no difference according to the net they fed through (χ^2=0.09, df=1, p=0.76), and no effects larval selection (χ^2=0.07, df=1, p=0.79), adult selection (χ^2=0.51, df=1, p=0.48), or the interaction (all p>0.22).

Among the females that laid eggs, those that took blood through an untreated net laid 29 % more eggs than females that bit through an Olyset Plus® net (145.2 ± 3.3 (95 % CI) against 112.9 ± 4.1; χ^2=143.96, df=1, p>0.001). We found no effect of larval selection or adult selection treatment alone (χ^2=2.46, df=1, p=0.12; and χ^2=0.16, df=1, p=0.69), but there was a trend for an interaction between both treatments (χ^2=2.81, df=1, p=0.093). A post-hoc analysis of this interaction showed that permethrin exposure at larval stage did not affect the number of eggs laid by mosquitoes that were selected through an untreated net (t-ratio=0.034, df=8.1, p=0.97), but did slightly increase the number of eggs laid by mosquitoes that were selected through an Olyset Plus® net (t-ratio=2.4, df=7.9, p=0.043; **Fig. 4a**).

Pairwise comparisons performed for both nets separately however showed that no treatment differed from the selection control (untreated net: all p>0.43; Olyset Plus® net: all p>0.19, **Fig. 4a**).

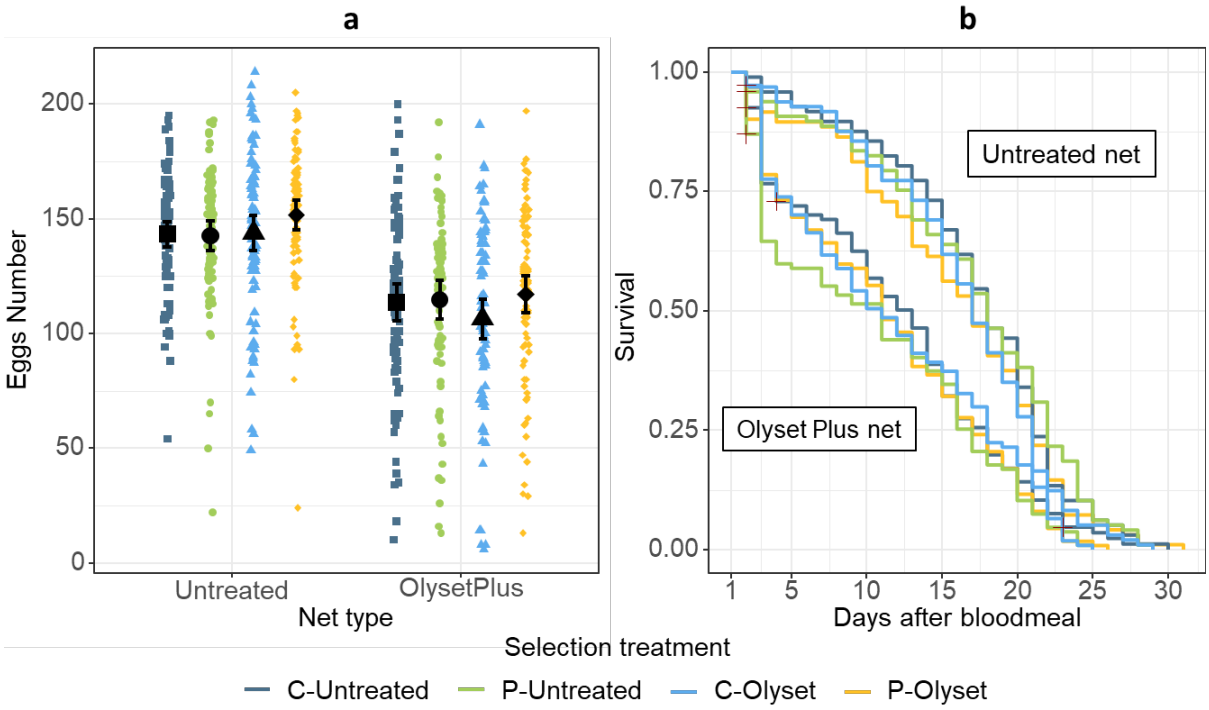

Figure 4. (a) Number of eggs laid per female (N=692) according the net they bit through (untreated or Olyset Plus® net) and the selection treatment they were applied (treatment abbreviations are described in the legend of Table 1). Error bars show the 95% confidence intervals. (b) longevity of mosquito females after the first 24 hours following a blood meal taken either through an Olyset Plus® net (lower curves) or through an untreated net (upper curves). In the order treatments are presented in the legend, N=108, 108, 109 and 112 (Olyset Plus® net); and N=97, 98, 97 and 96 (untreated net).

One day following the blood meal, 388 out of 389 (99.7% (95% CI: from 98.6 to 99.9%) mosquito females that took blood through the untreated net, and 437 out of the 490 (89.2% (95% CI: from 86.1 to 91.6%) that fed through an Olyset Plus® net were still alive (χ^2=14.59, df=1, p<0.001). Survival was however not affected by the larval or adult selection treatments (χ^2=0.00, df=1, p=0.95; and χ^2=0.65, df=1, p=0.42, respectively), and no interaction was significant (all p>0.34). Similarly, longevity following the first 24 hours after the blood meal was affected by the net mosquitoes bit through (χ^2=85.24, df=1, p<0.001), with the 50 % mortality threshold being reached ca. 5 days earlier for females that took a blood meal through an Olyset Plus® net, compared to those having fed through an untreated net (**Fig. 4b**). Longevity was not affected by the larval or adult selection treatments (χ^2=0.96, df=1, p=0.33; and χ^2=1.56, df=1, p=0.21, respectively), and no interaction was significant (all p>0.12).

7.4.5 Resistance level and mosquito traits

The correlations between the resistance level of mosquitoes (LT50) and their performance when tested on an untreated or Olyset Plus net are described in **Table 1**. Several traits were found to be correlated with LT50: biting success, egg number, 24h survival and longevity. We found a consistent a

trend for a positive correlation between resistance level and egg number irrespective of the net mosquitoes were fed through (**Fig. 5b**), but the direction of other correlations was dependent on the type of net. While mosquitoes' biting success and 24h survival after a blood meal through an untreated net did not vary with resistance level, both traits were found to increase with resistance level when mosquitoes were fed through an Olyset Plus® net (**Fig. 5a** and **5c**). In addition, after a blood meal through an untreated net, mean longevity decreases when resistance increases, but the opposite was found when mosquitoes were fed through an Olyset Plus® net (**Fig. 5d**).

Table 3. Summary of the LMMs performed to test the effect of resistance (estimated with the LT50 value) on the performance of each selection line during the final tests (divided in three categories: feeding behavior, fecundity and survival). F and p values are given for the two explanatory variables and their interaction. An asterisk indicates significance (<0.05), and a "+" symbol indicates a p value lower than 0.1. In case of significant correlation or trends, the direction of the effect is indicated.

	Trait	explanatory variable	F value	p value	Effect direction
Feeding behavior	*Biting success* (log10)	LT50	$F_{1,47}$=4.32	0.038*	*positive*
		Net type	$F_{1,47}$=77.09	<0.001*	
		LT50:Net type	$F_{1,47}$=3.19	0.074^{+}	
	Time to bite	LT50	$F_{1,47}$=0.03	0.87	
		Net type	$F_{1,47}$=47.78	<0.001*	
		LT50:Net type	$F_{1,47}$=0.00	0.99	
	Feeding success (log10)	LT50	$F_{1,47}$=0.74	0.39	
		Net type	$F_{1,47}$=161.41	<0.001*	
		LT50:Net type	$F_{1,47}$=0.31	0.58	
	Feeding time (Square root)	LT50	$F_{1,47}$=0.26	0.61	
		Net type	$F_{1,47}$=1596.15	<0.001*	
		LT50:Net type	$F_{1,47}$=0.01	0.93	
Fecundity	*Laying success*	LT50	$F_{1,47}$=0.59	0.44	
		Net type	$F_{1,47}$=0.03	0.86	
		LT50:Net type	$F_{1,47}$=0.09	0.76	
	Egg number	LT50	$F_{1,47}$=3.10	0.078^{+}	*positive*
		Net type	$F_{1,47}$=12.11	<0.001*	
		LT50:Net type	$F_{1,47}$=0.17	0.68	
Survival	*24h survival* (cubed)	LT50	$F_{1,47}$=3.27	0.071^{+}	*positive*
		Net type	$F_{1,47}$=73.28	<0.001*	
		LT50:Net type	$F_{1,47}$=3.55	0.059^{+}	
	Longevity (squared)	LT50	$F_{1,47}$=0.53	0.47	
		Net type	$F_{1,47}$=22.56	<0.001*	
		LT50:Net type	$F_{1,47}$=7.11	0.008*	

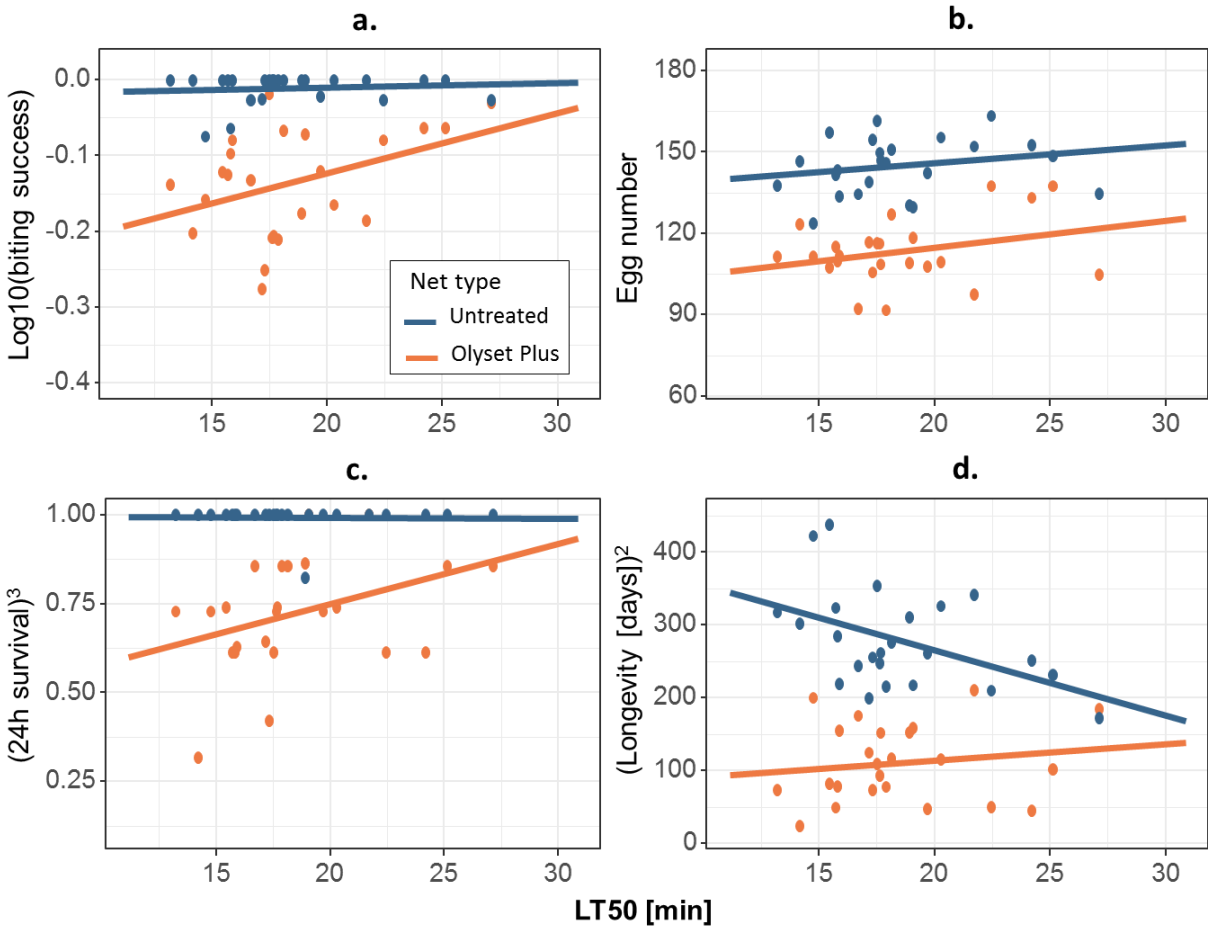

Figure 5. Linear regressions between the resistance level (LT50 values) of the selection lines and (a) biting success, (b) egg number, (c) survival after 24 hours following the blood meal, and (d) longevity, in function of the net mosquitoes were allowed to feed through (color legend). N=48 (12 lines x 2 blocks x 2 types of net).

Based on the regression coefficients, we also calculated the theoretical LT50 value that would allow mosquitoes to bite through an Olyset Plus® net and survive to it (24 h survival) with the same efficacy as unselected control mosquitoes would do through an untreated net. We found that an LT50 of 33 min 50 s would, in theory, allow mosquitoes to bite through an Olyset Plus® net with 97.4% success (i.e. similar to the selection control lines on an untreated net), and an LT50 of 33 min 16 s would allow them to reach 100% survival 24 h after a blood meal taken through that net.

7.5 Discussion

For insecticide-sensitive mosquitoes, feeding through a 2% permethrin and 1% PBO impregnated bed net has deleterious consequences for their reproductive fitness (299), which is expected to select for resistance. However, mosquitoes forced to feed through such a net for 11 successive generations did only evolve for a higher resistance if they were first exposed to a sublethal concentration of permethrin at larval stage. While selected mosquitoes performed no or only slightly better than sensitive ones to feed through an Olyset Plus® net and survive, we found evidences that a higher resistance level likely increases both mosquitoes' biting success through an Olyset Plus® net and their survival afterwards.

However, a low or moderate resistance may not allow mosquitoes to compensate the fitness costs induced by the blood-meal taken through the PBO-ITN.

7.5.1 Evolution of resistance

For mosquitoes that were both exposed to permethrin at larval stage and selected through an Olyset Plus® net at adult stage during 11 generations, LT50 at the end of the selection phase was 41% higher than that of unselected control mosquitoes. To our knowledge, this is the first example of an experimental evolution of resistance following the exposure of both a pyrethroid and a synergist. Our results moreover demonstrate that even at sublethal concentration, permethrin may favor the evolution of resistance. This may be explained by at least two mechanisms. First, it has been hypothesized that sublethal doses of insecticides may hasten the evolution of resistance because of their mutagenicity (360). By increasing the mutation rate, the insecticide would therefore increase the probability that beneficial mutations appear and be further selected. However, most mutations being deleterious (361), this more likely to apply to large populations and less in our experimental system. Moreover, the mutagenic potential of pyrethroids has been found to be low (362) or absent (363), making this hypothesis even less likely.

Second, larval exposure, may impose energetical costs to mosquitoes that increase the selection pressure. For example, it is known that an exposure to xenobiotics induces the over-expression of detoxifying and antioxidant related genes (60, 121, 123), which are potentially costly for the organism (101, 127). One particularity of these mechanisms is that the overexpression only lasts for some days (193), but larval exposure is known to still confer a higher tolerance to permethrin at adult stage (e.g. (122)). This suggests that once they are first activated, detoxifying mechanisms have a stronger activity when reactivated in the future. It follows that the costs associated to an early-life exposure may be higher when mosquitoes are re-exposed later in life, as it was the case in our experiment. However, while it may explain the interaction between larval exposure and adult selection, it does not entirely explain why mosquitoes only exposed at larval stage did not evolve resistance at all. One possibility is that the costs of a single exposure at larval stage is low enough so that mosquitoes could compensate, e.g. through an increase in food intake. Alternatively, this treatment may eventually lead to the evolution of resistance, but 11 generations were not enough to detect it.

Similarly, mosquitoes that were selected at adult stage but not at larval stage did not evolve resistance neither, despite we could show that a blood meal through an Olyset Plus® net strong fitness costs. PBO, by restoring the susceptibility of resistant mosquitoes, may have decreased the advantages granted by resistance and thus slowed down its evolution. Thus, selection pressure apparently was only strong enough in mosquitoes that were additionally reared with a sublethal concentration of permethrin, possibly because of the mentioned costs.

7.5.3 Effects of resistance on Olyset Plus® net efficacy

Our results confirmed previous results by showing that biting through an Olyset Plus® net increased the time mosquitoes took before they start probing, decreased their biting success, feeding success, feeding duration, and fecundity (299). In addition, we also found a decrease in mosquitoes' longevity, which confirms that a sublethal contact with an insecticide treated bed net may have long-term consequences on survival (160). In such cases, malaria transmission may be impeded by reducing the risk that mosquitoes live long enough to transmit the pathogen (75, 76).

Surprisingly, resistant mosquitoes selected both as larvae and as adults were affected in similar ways than unselected mosquitoes when given the possibility to feed through an Olyset Plus® net. Biting success and egg number were slightly increased in that treatment compared to mosquitoes selected only at larval or adult stage, but they did not differ from that of unselected mosquitoes. Moreover, mosquitoes from the resistant treatment performed similarly to unselected mosquitoes for every other trait measured. These results suggest that the ITN confer the same protection to both sensitive and moderately resistant mosquitoes. However, if resistant mosquitoes do not perform better than sensitive ones, then it is hard to explain why resistance evolved. To explain this apparent contradiction, we propose three non-exclusive hypotheses.

First, the level of resistance may have been too low to detect significant changes in their ability to bite through an Olyset Plus® net. Indeed, after 11 generations the LT50 value in the resistant treatment was only 41 % higher than that of unselected control mosquitoes, which may not be enough to induce clear and detectable changes in the measured traits. Second, it might be that resistance has evolved to respond to selection at larval and not at adult stage, and did not increase the performances of adult mosquitoes biting through an Olyset Plus® net because of the presence of PBO. This possibility implies that both selection treatments, when applied together, induced significant larval mortality. Even though this was measured only once during the selection process, larval mortality was marginal and, importantly, we did not find a significant increase in larval mortality when both selection treatments were applied. Thus, while our data provide no conclusive evidence supporting this hypothesis, it cannot be completely excluded that selection also acted on larvae. Third, it might be that variation in resistance between the selection lines have hidden any global effect of the treatment on the measured traits. This hypothesis is supported by the results of the regression analyzes in which the resistance level and performance of each line was considered. The obtained results also give a more accurate picture of the potential effects of resistance on the protection conferred by the Olyset Plus® net.

In particular, these analyzes showed that resistance level was probably associated with a higher biting success through the ITN, an increased survival 24 h after the blood meal and, to a lesser extent, an increased number of eggs laid (**Fig. 5**). They also give insightful information about the rate at which

resistance provides benefits for mosquitoes (i.e. the slope of the regression lines). For example, with an LT50 of ca. 33 min (which is twice the LT50 of unselected sensitive mosquitoes), resistant mosquitoes are expected to reach similar biting success and 24 h survival than unselected mosquitoes would have on an untreated net. Thus, despite these estimated values must be taken with caution due to the non-linear relationship of some of the analyzed traits outside the range of our data, resistance may not necessarily have to be high to provide significant benefits to mosquitoes.

These results suggest that resistance may affect the protection conferred by PBO-ITNs in two main ways. First, a higher biting success may decrease the personal protection provided by the net. This is particularly important as biting success is usually not considered in standard procedures (contrarily to feeding success). Second, by decreasing the mortality experienced by mosquitoes in the first 24 hours following the blood meal, resistance may decrease both personal and community protection. Indeed, if mosquitoes survive to the blood meal, they may try to bite again or leave to find someone else to bite.

In addition, resistance showed a trend towards a positive correlation with egg number. However, this positive association was also found for mosquitoes that bit through an untreated net. Thus, while resistance may benefit to mosquitoes biting through an ITN by increasing their fecundity, they will still suffer from a significant fitness cost compared to a blood meal taken through an untreated net. It follows that the ITN is expected to still provides a valuable community protection against resistant mosquitoes by reducing their number. That insecticide resistance may increase fecundity is, however, counterintuitive; the opposite being more frequently observed (see (364) for examples). Rare cases of positive associations have been reported with a DDT (organochlorine) resistant fly (365) and a malathion (organophosphate) resistant red flour beetle (366–368). However, the mechanisms behind these observations are largely unknown.

Importantly also, our results show that an increase in permethrin resistance was associated with a marked decrease in longevity when mosquitoes were fed through an untreated net (**Fig. 5d**). This show that even a moderate level of resistance may induce strong fitness costs in the absence of insecticide (101–103, 107, 369). These costs are likely to impose a major constraint to the evolution of resistance, especially if the selection pressure is not constant. Moreover, the rate at which resistance increased the longevity of mosquitoes biting through an Olyset Plus® net was low, suggesting that resistance level should be high to compensate for the fitness cost induced by the contact with that type of nets.

To conclude, we showed that a PBO-based ITN could contribute to the evolution of resistance in mosquitoes, but that larval environment was a key parameter of this evolution. Our results suggest that a sublethal exposure to insecticide at larval stage could act as a catalyzer and hasten the evolution

of resistance. In addition, we showed that a moderate insecticide resistance may decrease the personal protection conferred by the net by increasing the risk mosquitoes bite through it and reducing its short-term insecticidal property. However, Olyset Plus® net still contribute to the protection of the community by decreasing moderately resistant mosquitoes' longevity and reproduction. Thus, the global efficacy of PBO-based ITNs is likely to remain high even against low to moderately resistant mosquitoes (355).

Chapter 8

Synthesis

8.1 Summary of the results

8.1.1 Chapter 2

In this first experiment, we assessed the consequences of a sublethal exposure to permethrin at larval stage on mosquito development time, adult tolerance to permethrin, adult fecundity and longevity, as well as the fecundity and longevity of the progeny. In addition, we tested whether the hatching success of mosquito eggs was affected by the presence of permethrin in the water or by the sublethal early-life exposure of the mothers. Our results showed that permethrin at the tested concentration negatively affected development time, had no effect on adult size and longevity, tended to increase egg-laying success, and increased adult tolerance to permethrin. On the second generation however, mosquitoes whose mothers have been exposed lived less long. Also, we found that neither the presence of permethrin in water nor the exposure status of the mothers affected egg hatching success. We conclude that permethrin at low dose may induce a stimulatory response on mosquito fitness via an increased reproductive success, but that this stimulation may come at a cross-generational cost on longevity.

8.1.2 Chapter 3

In this chapter, we investigated the effect of permethrin exposure at larval and/or adult stage and the effect of larval competition on the response of mosquitoes to an infection with *Plasmodium berghei*. We found that both the prevalence of infection and infectiousness were ca. 20% lower in mosquitoes that were previously exposed to permethrin as larvae, adults, or both. Surprisingly, larval competition had no impact on infection prevalence or infectiousness but decreased the intensity of infection. In addition, mosquitoes reared under competition for food suffered from a higher mortality after they took the infectious blood meal. Permethrin exposure did help mosquitoes from the competition treatment to survive the infection, but the effect was only significant when mosquitoes were exposed at both larval and adult stages. We conclude from these results that permethrin exposure was mainly beneficial for mosquitoes as it helped them to clear the infection. This effect is also potentially beneficial for humans as it leads to a decrease of the proportion of infectious mosquitoes. The impact may, however, be lower in the case of high larval competition, as an exposure to permethrin may increase mosquitoes' survival and thus counterbalancing the effect it has on infection prevalence.

8.1.3 Chapter 4

In the fourth chapter, we tested the immune responses of mosquitoes that were exposed to permethrin at larval stage. The effect of larval competition for food was also tested alone or in combination with the insecticidal exposure. Adult mosquitoes were injected either with a bead to measure the ability of mosquitoes to melanize, or with *E. coli* bacteria to measure their antibacterial

response. We found that competition negatively affected the immune responses of mosquitoes by decreasing their melanization capacity (both the proportion of melanized beads and the amount of melanin deposited), and their antibacterial response. The effect of permethrin exposure was strongly dependent on whether mosquitoes were reared individually or in competition. When mosquitoes were reared individually, those that were exposed to the insecticide had a higher melanization success, deposited more melanin on the beads, and mounted a stronger antibacterial response. However, when mosquitoes were reared in competition, those that were exposed to permethrin had a higher melanization success but showed similar immune responses than unexposed mosquitoes otherwise. We conclude that a low dose of permethrin activates immune-related mechanisms, but that larval competition for food probably imposes a strong energetic constraint on these mechanisms. Hence, in nature, the net effect of permethrin residual may primarily depend on larval conditions.

8.1.4 Chapter 5

In this chapter, we investigated the possibility that a sublethal doses of permethrin and larval competition induce a global change on mosquitoes' oxidative state. The effect of permethrin exposure at both larval and/or adult stages was tested. In addition, we assessed whether differences in mosquitoes' oxidative balance could affect their ability to melanize beads. Surprisingly, we found that larval competition had no main effect on adults' oxidative state, and that permethrin exposure at larval stage resulted in a higher antioxidant response at adult stage. As permethrin is strongly pro-oxidant, we suggested that the observed antioxidant response in adult mosquitoes was due to a priming effect induced by the exposure at larval stage. In mosquitoes reared under competition for food, this effect was however only visible when insecticide was applied at both larval and adult stages, suggesting that a repeated exposure may increase or maintain the priming mechanism. Finally, we found that a high antioxidant defence was associated with a higher melanization capacity (higher amount of melanin deposited on the injected beads). As the melanization process induces oxidative stress, we proposed that a higher antioxidant defence allows mosquitoes to invest more into melanization. We conclude that permethrin at low dose may help mosquitoes to respond to further oxidative stress, which may be globally beneficial for them, especially regarding their immune response. Larval competition limits these benefits, suggesting that the priming mechanism may be energetically costly.

8.1.5 Chapter 6

To bite a person sleeping under an ITN, mosquitoes either have to find a hole to pass through, or bite directly through the net. In this chapter we tested this latter possibility using an PBO-ITN, the net Olyset Plus®. Unexpectedly, we found that a large proportion (ca. 70%) of mosquitoes were able to bite through the ITN, and that most of those who took a blood meal through that net survived (85%). We also showed that this surprisingly high survival was caused by the blood meal itself through an

unknown mechanism. We proposed two hypotheses, the temperature of the blood and oxidative stress, to help mosquitoes to survive the contact with the Olyset Plus® net. Finally, we found that mosquitoes were irritated by the net and left sooner after they start probing, which lead to a smaller volume of blood ingested and a lower fecundity afterwards. We conclude that mosquitoes that had the possibility to bite through an ITN might do so and had good chances to survive, but may pay a significant fitness cost by laying less eggs.

8.1.6 Chapter 7

In this last experiment, we tested whether biting through an Olyset Plus® net and/or being exposed to a sublethal dose of permethrin at larval stage could lead mosquitoes to evolve resistance to permethrin. We also evaluated whether the selected resistance increased the capacity of mosquitoes to bite through the ITN, survive to the exposure and lay eggs. We found that permethrin resistance only evolved in mosquitoes that were exposed at larval stage and fed through an ITN at adult stage. Larval exposure or a blood meal through the ITN alone did not select for higher resistance after the selection process. In addition, we found evidences that permethrin resistance may help mosquitoes to bite (but not feed) through a treated net and survive. However, our results also suggest that a high level of resistance is required to diminish the costs of such blood meal on fecundity and longevity. We conclude that larval exposure, despite being sublethal, could catalyze the evolution of resistance of mosquitoes that undergo a stronger selection pressure later in life. The insecticide may do so by inducing energetical costs that increase with repeated exposure. Also, despite resistance may slightly decrease the personal protection conferred by the net, it may not allow mosquitoes to easily compensate for the deleterious effect of the ITN on mosquitoes' longevity and fecundity. Thus, Olyset Plus® net may still effectively protect the community against moderately resistant mosquitoes by reducing the probability they live long enough to transmit the parasite, and by affecting their reproductive fitness.

8.2 Synthesis and further perspectives

8.2.1 Chapter 2-5

To sum up, our results show that at sublethal (or nearly sublethal) concentration, permethrin was mostly beneficial for mosquitoes. Mosquito females that were exposed to the insecticide at larval stage had a higher fecundity, a higher immunocompetence (increased melanization and antibacterial responses), an increased antioxidant capacity, and were more tolerant to permethrin at adult stage than unexposed mosquitoes. When infected with *Plasmodium berghei*, exposed mosquitoes also had a lower infection prevalence and infectiousness, and benefitted – in some cases – from a higher survival after the infection. These results support the idea of a hormetic response of mosquitoes

exposed to pyrethroids, with a stimulatory response at sublethal concentration and detrimental or lethal effects at higher concentration (370–372). In the different chapters, we discussed several possible mechanisms involved in the stimulation of these traits after an exposure to the insecticide. For most traits investigated, oxidative stress was one of the potential mechanisms involved.
Indeed, permethrin is known induce OS (270), to which the organism has to respond. This antioxidant response and the plastic modulation of the oxidative state later in life is a major physiological consequence of the exposure to permethrin. Importantly, OS and the antioxidant capacity are, in turn, associated to mosquito fecundity (145), tolerance to insecticides (210, 317), melanization capacity (Chapter 5 and (154)), and other immune-related mechanisms (148, 153) including against bacteria and *Plasmodium* (150). Therefore, even though our results suggest that OS is probably not the only mechanism involved, we found conclusive evidences showing that it may be one of the main mechanisms through which insecticides affects so many different mosquito traits. These results globally supports the view of Costantini, who proposed OS to be one of the main driver of hormesis (373), but also one of the cause of the deleterious effects observed when the level of stress increases. Indeed, the cost:benefit ratio is expected to reverse when the stress increases, because a high level of oxidative stress may in turn have detrimental consequences on an organism's life history (146).

In the present work, we also investigated the role of larval competition. Our results highlight the fact that the benefits provided by the insecticide are often dependent of larval nutrition, and are thus probably energetically costly. This was also highlighted in Chapter 2, as the positive effect of larval exposure on fecundity induced cross-generational costs on longevity. In addition to resource availability, other stressors are known to interact with insecticides, such as temperature (189, 346), the presence of predators (215) or pathogens (374–376). Thus, while it has been argued that hormesis is a general mechanism that should be observed in many situations (68, 377), the consequence of a single dose of insecticide may still greatly vary according to the studied system and the environmental conditions.

The implications of our results for the vectorial capacity of mosquitoes for malaria parasites is, overall, difficult to predict. On the one hand, the increase in fecundity may results in higher densities of mosquitoes per host, and thus an increase in vectorial capacity. Also, an increased tolerance to insecticides at adult stage may, to some extent, reduce the efficacy of mosquito control tools. On the other hand, the cross-generational costs on longevity and the reduced malaria prevalence may lead to a strong decrease in malaria transmission. Immunity here has a key but complex role, as a stronger immune defense may both increase mosquitoes' survival and decrease malaria prevalence, as observed in Chapter 2. While the net outcome may depend on the particular situation of interest, the

present work highlights the various ways through which insecticide residues may affect vectorial capacity and stresses the need for a deeper understanding of the mechanisms involved.

Finally, as our results show that permethrin at low dose induce important fitness variations, selection may favor mosquitoes that benefits the most from the exposure. This will eventually lead to the evolution of resistance if 1) this variation in fitness has a genetic component (as suggested in Chapter 2), and 2) if it is genetically correlated to resistance mechanisms. While these points were not properly tested, our findings provide some indirect evidences supporting this idea. In particular, oxidative stress may be both involved in the stimulatory response observed (as discussed above) and in the resistance to insecticides (122, 147, 317). This hypothesis has strong implications for the evolution of resistance and would deserve further investigations.

8.2.2 Chapters 6 and 7

In Chapter 6, we disentangled the various ways through which an ITN affect the behavior and fitness of mosquitoes that have the possibility to bite through the net. Importantly, we found that even if mosquitoes succeed to take blood, they will still suffer from significant fitness costs. Thus, the ITN contribute to decrease mosquito population even when it fails to prevent from mosquito bites. However, these results also highlight the risk for people protected by ITNs to touch the net, as the insecticide is not irritant or repellent enough to prevent all mosquitoes to bite through. Furthermore, our findings also have broader implication for insect physiology and pyrethroids toxicology, as we showed that a blood meal may strongly decrease the mortality induced by the chemical. This latter result was unexpected and, to our knowledge, has never been investigated before. Because of its potential implications, it may be worth for future research to look into the mechanisms involved, possible oxidative stress and/or temperature variation during the blood meal.

Chapter 7 expands some of these questions by showing that insecticide resistance may partially impede the personal protection conferred by the tested ITN, but that it still confers a high community protection by decreasing the fitness of low to moderately resistant mosquitoes. Importantly, this experiment also demonstrated that an insecticide at sublethal concentration can favor the evolution of resistance. To our knowledge, it is the first evidence that a non-selecting dose of insecticide increase the speed of the evolution of resistance to insecticides. Whether it is a general feature of insecticide residues is unknown, and we lack a clear view of the interactions they might be between the initial a sublethal exposure and the lethal selecting dose on the fitness of the mosquitoes. Further studies should address these points to have a deeper understanding of selection process leading to the observed results.

8.3 Conclusion

This work has investigated the effects of an insecticide at sublethal concentration on a wide range of behavioral, physiological, and fitness traits of the malaria mosquito *Anopheles gambiae*. An important result of this thesis is that at low dose, the insecticide was mostly beneficial and not detrimental to mosquitoes. Moreover, the various effects observed suggest that the consequences of such low doses on mosquito's vectorial capacity may be as important as with high doses. In addition, we also demonstrated experimentally that a sublethal dose of insecticide could favour the evolution of insecticide resistance in mosquitoes, with a potential impact on the efficacy of vector control tools. Finally, we showed that larval nutrition had a key role in the response of mosquitoes to insecticide, which points out the sensitivity of our results to environmental conditions. Altogether, our results contribute to a broader understanding of the eco-toxicological consequences of insecticide residues, and underline the necessity for a better consideration of these effects in the epidemiology of vector-borne diseases.

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