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Ecological immunity of *Anopheles gambiae* mosquitoes

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Abstract

Malaria is a vector borne disease caused by protozoan parasites and transmitted to humans by mosquitoes. It is one of the most deadly infectious diseases in the world, with more than one million deaths each year. Malaria is even more worrying in the context of climate change, as global warming is predicted to increase the transmission to humans and to influence vector-parasite interactions. To answer these concerns, a better understanding of the ecological immunity of mosquitoes is needed, as it takes into account the intrinsic and environmental factors that may affect the mosquito vector competence. This evolutionary ecology approach relies on the correlations between the immune responses and other life history traits key to the fitness of the mosquito - eg. longevity, reproductive success, size. To improve our knowledge of the ecological immunity of mosquitoes, we decided to study the variability in immune responses and the influence of environmental factors on malaria transmission by *Anopheles gambiae* mosquitoes in the laboratory.

We first investigated the variability in the resistance to malaria parasites, as some mosquitoes become infectious and transmit the parasite, despite an efficient immune system. We focused on two major immune effectors the encapsulation of parasites with melanin and their lysis by antimicrobial peptides. Both the melanisation response and the antimicrobial response were physiologically costly in *A. gambiae*, with a reduction of the lifespan after an immune challenge. Besides, we found also negative genetic correlations between the costs of the two immune responses and between the cost and the efficacy of the melanisation response. In addition, the melanisation response could be overloaded by an increasing immune challenge, with an unequal repartition of the melanisation effort. Mosquito immunity is made of costs, physiological limits and complex interactions between benefits and costs that will determine the evolution of immunity and the resistance to malaria parasites.

Environmental factors also may influence the vector competence of mosquitoes, as there is a direct effect on adult traits - eg. longevity, reproductive success. There is also an indirect effect because of the influence on the larval development with consequences on adult traits. The interaction between the

larval diet and the temperature may influence the probability that mosquitoes infected with the malaria parasite survived up to harbour sporozoites (the infectious stage of the parasite) in their salivary glands. Undernourishment leads to a lower parasite and mosquito survival. Temperature on the other hand, had just an effect on parasite survival in interaction with the larval diet, as a low temperature was in favour of malaria transmission with a standard diet. The association between mosquito size and longevity was also dependent on environmental factors during larval development. The relationship was null at 25°c, it was negative when the environmental factors had opposed effects on size, and positive when they had synergic effects. The larval ecology influences the vector competence and as a consequence malaria transmission.

The ecological immunity of mosquitoes may lead to a more complex vision of the evolution of immunity. Besides, environmental factors will also influence the resistance of mosquitoes to malaria parasites and malaria transmission. These results confirm the importance of improving further our understanding of host-parasite interactions for efficient vector control programs.

Key words: mosquito**,** malaria transmission, immunity, melanisation response, antimicrobial response, longevity, size, environment, temperature, food

Résumé

Le paludisme est une maladie infectieuse causée par des parasites protozoaires et transmise à l'homme par la piqûre de moustiques infectés. Avec plus d'un million de morts chaque année, c'est l'une des maladies infectieuses les plus mortelles au niveau mondial. Le changement climatique rend l'impact de cette maladie d'autant plus préoccupant. En effet, le réchauffement global risque d'augmenter la transmission du paludisme et de modifier les interactions entre le vecteur et le parasite. L'éco-immunologie des moustiques est un moyen de répondre à ces inquiétudes, car elle prend en compte les facteurs intrinsèques au moustique et les facteurs environnementaux qui peuvent influencer sa compétence vectorielle. En effet, cette approche d'écologie évolutive a pour but d'étudier les corrélations entre l'immunité du moustique et les autres traits d'histoire de vie essentiels à la détermination de la fitness du moustique - durée de vie, succès reproductif, taille. Pour améliorer notre compréhension de l'éco-immunologie des moustiques, nous avons décidé d'étudier la variabilité de la réponse immunitaire et l'influence des facteurs environnementaux sur la transmission du paludisme par *Anopheles gambiae* en laboratoire.

Tout d'abord, nous avons étudié la variabilité de la résistance du moustique face aux parasites du paludisme, certains devenant infectés malgré la présence d'un système immunitaire fonctionnel. Nous nous sommes concentrés sur deux réponses immunitaires majeures: l'encapsulation des parasites avec de la mélanine et la production de peptides antimicrobiens qui vont tuer les parasites. La mélanisation et la réponse antimicrobienne sont coûteuses pour le moustique avec pour conséquence la réduction de la durée de vie. Nous avons également observé des corrélations génétiques négatives entre les coûts des deux réponses immunitaires et entre le coût et l'efficacité de la mélanisation. En outre, la mélanisation est une réponse qui peut être rapidement surchargée par l'augmentation de la stimulation immunitaire. L'immunité du moustique est donc conditionnée à des coûts, des limites physiologiques et un ensemble complexe de corrélations entre réponses immunitaires et coûts, qui vont déterminer son évolution et pourrait limiter la résistance au paludisme.

Les facteurs environnementaux peuvent eux aussi influer sur la capacité vectorielle d'*A.gambiae*, et ce directement, en modifiant les traits d'histoires de vie des adultes, tels que la longévité ou la fécondité. Ces facteurs ont aussi un effet indirect par leur influence sur développement larvaire avec des conséquences pour l'adulte. L'interaction entre le régime alimentaire des larves et la température peut modifier la probabilité qu'un moustique infecté par le parasite du paludisme survive jusqu'à avoir des sporozoites (le stade infectieux du parasite pour l'homme) dans ses glandes salivaires. La malnutrition conduit à la diminution de la survie du parasite et du moustique. La température quant à elle, a seulement un effet sur la survie du parasite et ce selon le régime alimentaire des larves. Les basses températures favorisent la transmission du parasite mais ce seulement avec un régime alimentaire standard. La relation entre la taille du moustique et sa longévité dépend elle aussi de l'environnement des larves. La relation est inexistante à 25°C, négative quand les facteurs environnementaux ont des effets opposés sur la taille de l'adulte, et positive quand ils ont des effets synergiques sur la taille. L'écologie des larves influence la compétence vectorielle de l'adulte et sa fitness et donc par conséquent la transmission du paludisme.

L'étude de l'éco-immunologie du moustique nous amène à développer une vision plus complexe de l'évolution de l'immunité. De plus, les facteurs environnementaux vont aussi influencer la résistance du moustique face au paludisme et la transmission de cette maladie à l'homme. Ces résultats confirment la nécessité de continuer à améliorer notre compréhension des interactions entre le vecteur et le parasite pour développer des programmes de lutte vectorielle plus performants.

Mots clés: moustique, transmission du paludisme, immunité, mélanisation, réponse antimicrobienne, longévité, taille, environnement, température, nourriture

Contents

Chapter 1

1. General introduction

1.1 Background

Malaria burden

Malaria is a vector borne disease caused by protozoan parasites and transmitted to humans by mosquitoes [1]. Malaria concerns more than 500 000 millions people in the world [2, 3] and it is one of the first causes of death by infectious diseases [4], with more than 1 million deaths each year [2, 5]. It is a major burden for pregnant women [6] and children, mostly in Sub-Saharan Africa [4] but also in Asia and South America [2, 5]. Children, under 5 years old, are the most at risk and even more under 2 years old, when they have yet to acquire clinical immunity [3, 7], as malaria provokes acute febrile illness, chronic repeated infections with long-term consequences and eventually death, if not treated [8]. The associated morbidity is a strong economic burden on the poorest populations [4, 9].

Malaria transmission

Plasmodium parasites are responsible for this infectious disease in vertebrate hosts [1] and the five human parasites species are *Plasmodium falciparum*, which is the most virulent, *P.vivax*, *P.ovale, P.knowlesi* and *P.malariae* [10]. *Anopheles* mosquitoes are the major vectors of these parasites, with quite specific associations between vector and parasite species [1]. *Anopheles gambiae s.s.,* an anthropophilic and endophilic vector, bites humans inside their houses at night and transmits *P. falciparum* [11–14]. *A. gambiae* females are anautogenous animals, as they need a blood meal to produce mature eggs before laying them in aquatic environments [15–18], where the larva will hatch and transform in a pupae, before emerging as an adult [19].

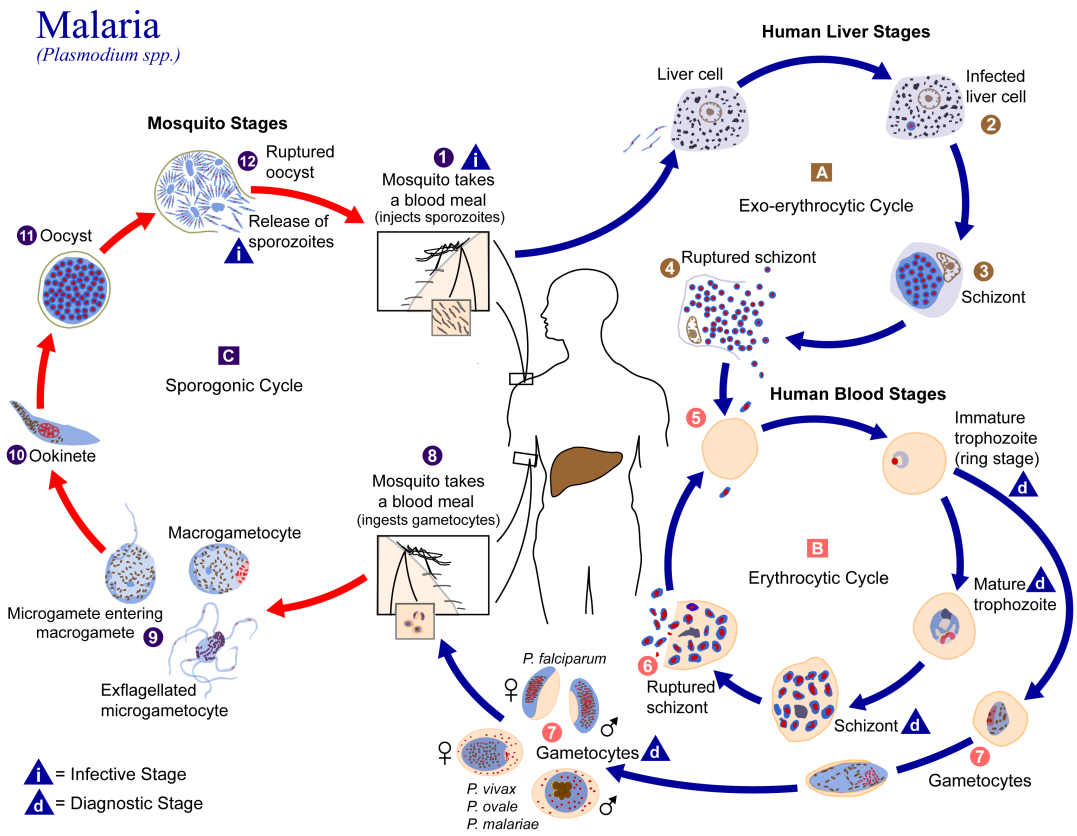

Figure.1 Malaria life cycle and transmission (reproduced from the CDC website).

The sporogonic life cycle in the mosquito is about 14 days long (C). After an infectious blood meal, the ingested *Plasmodium* gametocytes differentiate in the mosquito midgut and undergo a fusion to produce a zygote. About 20 hours later, the zygote becomes an ookinete that will invade the midgut epithelium of the mosquito, reach the basal part close to the haemolymph, and start forming oocysts. These oocysts contain up to 8000 sporozoïtes at maturity that will be released in the haemolymph, about 14 days after the blood meal. Transported by the flow, the sporozoites may then reach and invade the salivary glands [20, 21]. Once in the salivary glands, parasites can be transmitted to humans during the next blood meal, as the saliva acts as an anticoagulant during mosquito feeding [22]. Inside humans, the sporozoites may reach the liver thanks to the bloodstream and hide in the hepatocytes (A). Once the parasites have formed schizonts and then differentiated to mature merozoïtes they may be released back to the bloodstream and infect red blood cells (B). They will reproduce asexually and destroy the blood cells, while some of them will become gametocytes that can be transmitted to mosquitoes [23, 24].

Antimalarial treatments

Efficient antimalarial drugs have been developed against infections in humans, like chloroquinine or artemisinin (part of the Atermisinin Combination Therapy (ACTs)) [25]. However, there is increasing parasite resistance against antimalarial drugs [26–29], even against the most recent drugs, like artemisinin [30]. In addition, antimalarial drugs are quite expensive while malaria is mostly a threat in developing regions with poor populations [26], associated to morbidity, it is part of the economic burden that malaria represents. Furthermore, many studies were also conducted to develop a vaccine against malaria parasites since more than fifty years [31, 32], but none has yet been proven sufficiently efficient to be an alternative to antimalarial treatments [33, 34].

Vector control

Vector control complements the antimalarial treatments, especially in areas with a difficult access to drugs. The classic tools of vector control against endophilic mosquitoes like *A. gambiae* are the Insecticide treated nets (ITN), under which people sleep, and the Indoors residual spraying (IRS) of insecticides on the walls of their houses. The destruction of breeding sites is preferred against exophilic mosquitoes [35, 36]. ITN and IRS prevent infected mosquitoes to blood feed on humans at night and to transmit the parasite [37]. Their second goal is to prevent uninfected mosquitoes to get infectious blood meals and to kill them before malaria gets transmitted to humans [37, 38] upon further blood meals [21, 39]. Pyrethroids are the major class of insecticides used on these bed nets [36] but mosquito populations are developing resistances against these insecticides [35, 40]. Some alternative tools are under development, like the release of sterile males [41] or the release of genetically modified mosquitoes resistant to malaria parasites [42–44]. Sterile males are supposed to compete with wild males for mating while leading to sterile descendants [45], the goal is to reduce mosquito populations around towns and villages [41]. The genetically modified mosquitoes on the other hand are supposed to decrease malaria transmission by limiting the number of infectious mosquitoes and so the number of infectious bites for human [42, 44].

Vector intrinsic factors, modelling transmission

Many models have been designed to predict malaria transmission [46–48] since the classic Ross-Macdonald model, which gives the basic reproductive number for malaria, R_0, and the potential for the spread of malaria [49]. In such models, there is a large emphasis on vector parameters like mosquito density, biting rate and longevity [46, 49]. These parameters are the aim of vector control tools and key to reduce malaria transmission [46]. Mosquito longevity for example is key to the completion of the sporogonic cycle and most infected mosquitoes will die before the end of the parasite development in the wild [37]. The parasite has indeed negative consequences on the mosquito's fitness, as it decreases mosquito fecundity [50–52] and survival [53, 54]. Mosquito infectiousness is essential too [55] and the resistance to malaria parasites is variable [56–58]. Knowing the importance of the variation in mosquito longevity and infectiousness for the modelling of malaria transmission [59], we defined here vector competence as the probability that *Anopheles gambiae* mosquitoes infected with the malaria parasite survived up to harbour sporozoites (the infectious stage of the parasite) in their salivary glands.

Mosquito immunity

Mosquito infectiousness is crucial to the vector competence and it is influenced by the mosquito immune system, as malaria parasites encounter different bottlenecks during their development in the mosquito [55]. Parasites have first to get past the physical barriers of the cuticle and the midgut epithelium [60] before entering the haemolymph circulating system and reaching the salivary glands. Immunes responses in the different compartments of the mosquito body are efficient against malaria parasites [61–63] but also against bacteria [64], filarial nematodes [65] and viruses [66].

In the midgut, the cibarial armature can crush large parasites like filarial worms and the peritrophic matrix, that develops after a blood meal, is an extra hurdle before the midgut epithelium [60]. Parasites may also be detected by the pattern recognition receptors (PRR), as self and non-self recognition is the first essential step of the immune response [67]. Parasites stimulate the immune system by damaging host cells (release of collagen and nucleic acids) or secreting molecules

that interfere with the host biology like PAMPs (pathogen-associated molecular patterns) [68], as lipopolysaccharides (LPS) or peptidoglycan (PGN) [69–71]. The main immune signalling pathways induced by the parasites are Imd, Toll, the JAK/STAT [72], and the RNAi (Ribonucleic acid interference) pathways [73, 74], (Figure 2).

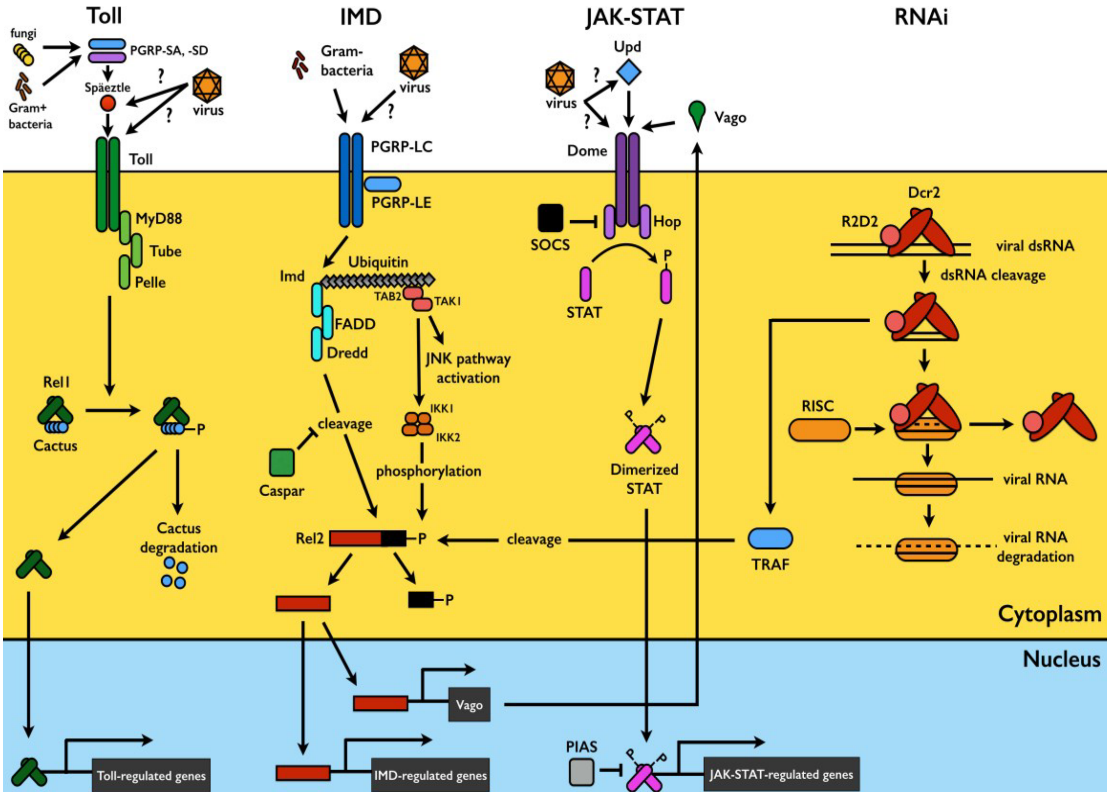

Figure 2. Anopheles gambiae immune signalling pathways and key genes (adapted from [75]). The Imd pathway is involved in defence against bacteria, viruses and Plasmodium [76]. The Toll pathway is induced by viruses, Plasmodium, Gram (+) bacteria and fungus [76]. Both Imd and Toll are efficient against Plasmodium ookinetes and result in the production of Antimicrobial peptides (AMP) and phagocytosis [75]. The JAK/STAT is induced by bacteria, Plasmodium and viruses and result in the production of nitric oxide synthase (NOS) and reactive oxygen species (ROS) that lead to the lysis of Plasmodium ookinetes and oocysts [55, 76] and infected cell apoptosis. The RNAi is induced by the detection of long virus dsRNA by RNase in mosquito cells [73]. It results in RNA silencing to prevent virus multiplication.

These immune signalling pathways result in the production of effectors [20, 63, 64, 67, 76–78] and both humoral and cellular responses [72]. AMP, ROS and NOS

result in the lysis of parasites, their phagocytosis or the apoptosis of infected cells, in the midgut and in the haemolymph (Figure 3) [76]. Coupled to these immune signalling pathways, you have the pro-phenoloxidase cascade (Figure 4), which results in the melanisation of parasites in the midgut and the haemolymph (Figure 3) and the production of cytotoxic compounds [79, 80].

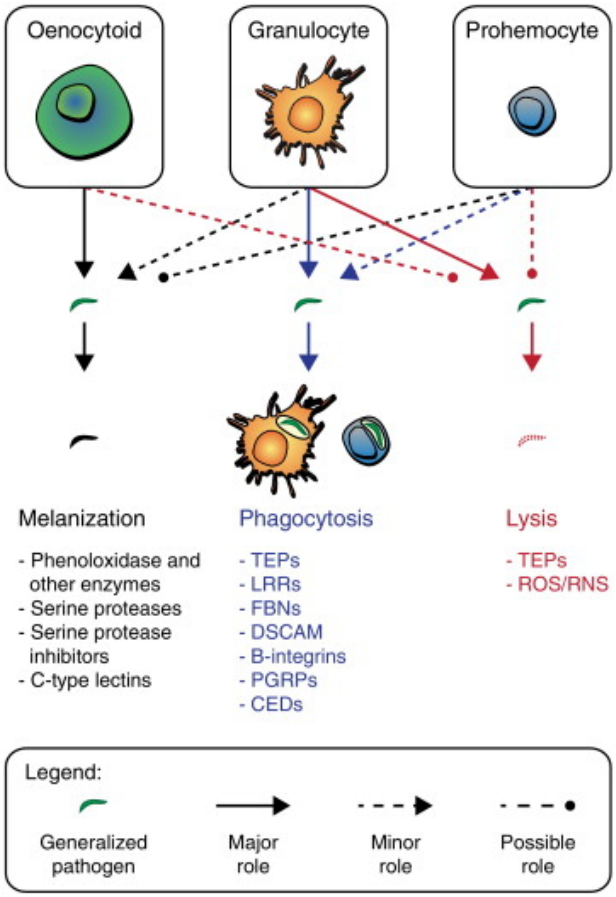

Figure 3. *Anopheles gambiae* immune effectors (modified from [81]). The three types of haemocytes immune cells (oenocytoid, granulocyte and prohaemocyte) and the fat body (not figured here) enable the production of immune effectors: melanisation, the phagocytosis and the lysis of parasites by antimicrobial peptides.

First, the melanisation response is humoral and consists in the production and deposition of a dark layer of melanin encapsulating parasites in the haemolymph. It can lead to their death or their phagocytosis by granulocytes [79, 80]. There is also the production of cytotoxic and oxidative compounds [80] during this biochemical pathway that converts tyrosin to melanin and start with

the pro-phenoloxydase protease cascade [79, 80], (Figure 4). Melanisation is implied in immunity, wound healing and egg production.

Second, mosquito haemocytes are able to phagocytise bacteria and small parasites by internalising them and digesting them [81, 82].

Third, the antimicrobial response results in the production of anti microbial peptides (AMP) by haemocytes and the fat body [81, 83], that will bind to parasites and kill them [20, 84]. For example, the family of Thioester-containing proteins (TEPs), and namely TEP1, can bind to the surface of gram-positive and gram-negative bacteria and Plasmodium ookinetes [1, 20, 78]. The knockdown of the TEP1 leads to a fivefold increase of parasitism in susceptible mosquitoes [63]. AMP could be efficient at killing malaria parasites like with the complex formed by TEP1, LRIM1 (leucine rich-repeat immune protein 1), and APL1A (leucine-rich repeat gene) in the hemolymph [21].

Melanisation and phagocytosis are fast immune responses (seconds to minutes) while the production of anti-microbial peptides (AMPs) is much slower (hours) [85] and could target the parasites that survived the constitutive immune response and persist in the insect body [86].

Finally, you also have antiviral responses in the mosquito haemolymph with RNAi against arthopod-borne viruses (arboviruses), as it will prevent the replication of virus RNA in the mosquito cells and regulate the development of virus inside mosquitoes [73, 74]. RNA silencing is the result of the RNA-induced silencing complex (RISC) that will degrade one strand of the dsRNA and keep the second one for detection or further ones [73, 74]. The other immune pathways may also have antiviral actions and in the mosquito midgut, the microbiota assures most of the antiviral defence [66].

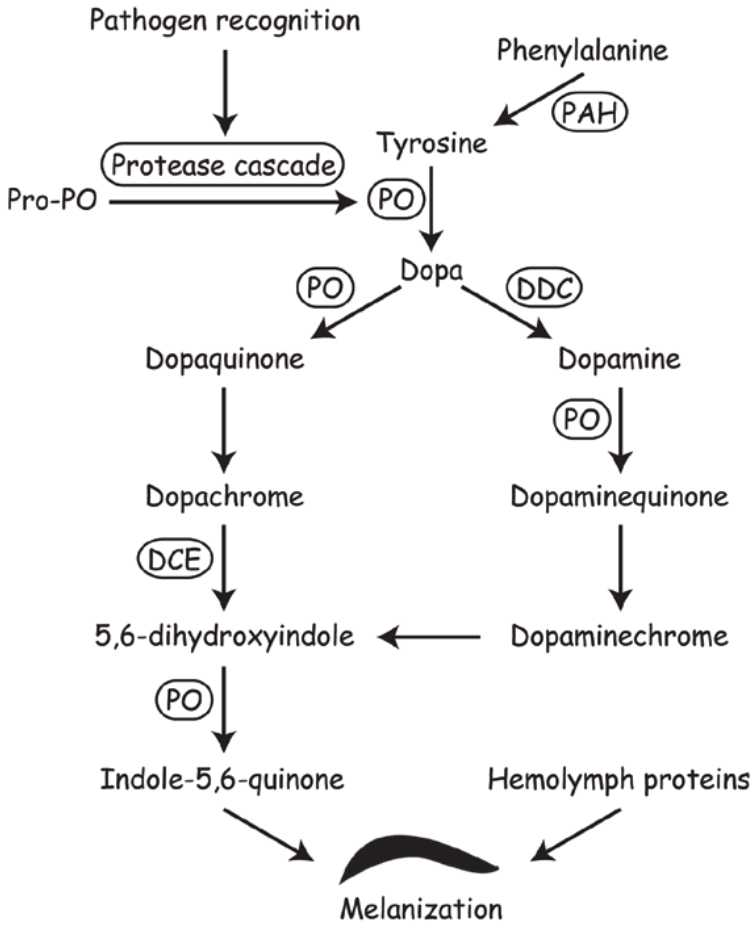

Figure 4. Melanisation cascade (modified from [76])**.** The key enzymes implied in the production of melanin for the melanisation of parasites are: PAH, phenylalanine hydroxylase; PO, phenoloxidase; DDC, dopa decarboxylase; and DCE, dopachrome conversion enzyme.

To sum up, in the case of malaria parasites, oxidative compounds (NOS and ROS) can kill ookinetes in the midgut. Ookinetes can also be killed by antimicrobial peptides, while invading the midgut epithelium cells, or be lysed during the apoptosis or the extrusion of these infected cells. If ookinetes still manage to reach the basal part of the midgut, close to the haemolymph, there can be phagocytosis or melanisation of the ookinetes and the oocysts. What happens to sporozoites in the haemolymph and the salivary gland is less well known [21, 84, 87–89].

Microbiota and immunity variability

The variability of the immune responses is in part linked to the bacterial species richness of the midgut [90], as bacteria trigger antimicrobial responses in the midgut and enhance the protection against parasites [91–94]. For example, there is a higher *Plasmodium* infection rate in *A.gambiae* mosquitos when the commensal bacteria are cleared by antibiotics and it is restored by adding such bacteria in a cleared midgut [92]. There are also essential antiviral defences in the midgut preventing entrance of virus in the haemolmyph [66]. The mosquito midgut is a dynamic environment from aquatic immature stages to terrestrial blood-feeding adults [95] and this microbiome may be the result of important selection pressures over the developmental time [96].

Immune evasion by parasites

Whatever the efficiency of these immune responses and of the microbiota some parasites are still able to evade the immune system [97–101] or supress it [100, 102, 103]. *P.berghei* for example, a rodent malaria parasite, acts on the regulation of several genes of *A. gambiae's* immune system [104] and suppresses the melanisation response [102]. Pathogen recognition is essential to trigger immune responses [67] and an high investment in the detection of damaged host cells or the pathogen-associated molecular patterns (PAMPs) secreted by parasites [68, 70, 71] may be costly for mosquitoes. Parasite evasion and immunosuppression and the energy needed for the detection of parasites could explain part of the immunity variability.

Costs of immunity

Another explanation to the variability of immunity is the costs of the immune responses. Indeed, there are benefits for the host of clearing the infection through the immune system to prevent detrimental effects of parasitic infection and the genetic basis of theses benefits is quite well known [67, 76, 105]. However, a maximal immune system doesn't mean an optimal one because there are constraints of having an efficient immune system [67, 106] and these costs can be physiological or genetic.

First, the physiological costs can be studied by looking at phenotypic correlations, through experiments where the immune response is induced. For example, immuno-challenged bumble-bees have a shorten life-span under starvation and similar negative effects of immune stimulation and deployment have been observed in different invertebrate models, with a shorter lifespan or a smaller reproductive success [67, 107–113].

Second, the genetic costs are the negative genetic correlations between immune responses and other life history traits when there is no infection [67, 111], while comparing different genetic backgrounds. For example, a refractory strain of *A.gambiae* resistant against *Plasmodium yoelii nigeriensis* had a lower population growth rate than susceptible mosquitoes [112] in the absence of the parasite. Or, the artificial selection for resistance of *D.nigrospiracula* against ectoparasites leads to decreased fecundity of females in absence of parasitism [109].

These costs should be studied in regards of the different compartments of immunity. Decline in some aspects of an insect's immunity doesn't mean that the overall immunity is jeopardized. There may be a shift of allocation to another immune mechanism [113, 114] and different kind of correlations have been observed in mosquitoes [115] and in other insects [116–118]. A better understanding of these physiological and genetic costs could lead to improved knowledge of the variability and the evolution of immunity in mosquitoes.

Linked to these costs of immune responses, there can be damage caused to a host directly by its own immune system, called immunopathology [119, 120]. This is due to wrong parasite-killing mechanism, oversized immune responses or eliciting by the wrong antigens [119, 120].

Environmental factors, immunity, hosts and parasites traits

Environmental factors – e.g. humidity, temperature and food availability – affect mosquito immune responses and are an other source of variability [121–125]. There is an increase in Nitric oxide synthase with temperature but a decrease in phagocytosis [126] and melanisation [121]. There is also a better melanisation response when females have had a blood meal and sugar solution [123] or when they were fell fed as larvae [121]. The production of haemocytes and the antimicrobial response may also depend of larval nutrition [124]. Environmental

factors influence also other vector life history traits key to the mosquito fitness, as longevity is for example negatively correlated with the temperature [127]. Food availability also affects the longevity [128, 129] with an additional effect on the infection load [130]. Furthermore, environmental factors have also direct effects on the malaria parasites, as the parasite development rate increases with temperature [131–133] but with a decrease in survival [131, 134]. *Plasmodium* development inside the mosquito is possible in a wide range of temperatures from 16°C to 35°c [132] and host parasite interactions may then be highly variable along this range of temperatures.

Climate change and malaria transmission

Climate change is predicted to modify several of these environmental factors that influence host parasite interactions [135, 136] and it may enhance the risk of malaria transmission [131, 136–140]. Many studies showed a potential increase, or at least a variation, in malaria transmission with the temperature [122, 135, 138, 139, 141, 142], with most influence at the extremes temperatures of malaria transmission [138], linked for example to an augmentation of the transmission season [137]. So, climate change may directly influence mosquitoes and parasites traits through variations in environmental factors like rainfall, humidity [136, 143, 144], wind speed [136] and temperature [131, 138, 139, 141, 142]. Climate change may also influence malaria transmission through variations in mosquito population size [136, 138] and variations in mosquito, parasite and human behaviour [136], which could imply more encounters of infected vectors and human hosts.

1.2 Thesis introduction

Ecological immunity

A way to sum up all these ideas about hosts-parasites interactions in the environment is to study the ecological immunity of mosquitoes, as it takes into account the intrinsic and environmental factors that may affect the mosquito vector competence. This evolutionary ecology approach is based on the genetic correlations between immune traits and other life history traits [145, 146], and

the consequences for the evolutionary fitness of an individual in a varying environment [60, 67, 147]. The first studies were based on the large immunology knowledge in vertebrates [147] and then moved to invertebrates, first to *Drosophila melanogaster* and then to insects implied in vector borne diseases [60, 148]. These approach leads to a new general vision of immunity, as an optimum immunity rather than a maximum one [67, 149].

Despite the known importance of the vector for the epidemiological modelling and the control of malaria transmission [37, 43, 150], there is still a need to improve the growing knowledge on the ecological immunity of mosquitoes. We decided to study the immune responses variations in mosquitoes and the influence of two major environmental factors, temperature and food availability, on adults and larvae. A first part, was centred on host parasite interactions in controlled environments, as we were interested in getting a better understanding of mosquito immunity, linked to malaria resistance [59]. In the second part, we focused on the interactions between host and parasite traits in a varying environment, to study the mosquito vector competence and malaria transmission [151].

Genetic cost of inducing immunity and resistance to malaria parasites

Despite an efficient immune system [76], there is some variability in the mosquito's resistance to malaria parasites, as some we still become infectious and transmit the parasites. An explanation to this variability could be the antagonistic pleiotropy and the cost of the immune responses [60, 108, 148, 152]. This cost could be physiological or genetic. There are indeed physiological costs of inducing immune responses in mosquitoes [52] similar to the decrease in survival observed in immune challenged bumble bees [108]. Besides, there are genetic costs of maintaining an immune responses, it is to say negative genetic correlation between immune traits and other life history traits [112]. The evolution of immunity is a balance between the genetic costs and benefits of clearing a parasitic infection [60, 153].

There could also be genetic cost of inducing an immune response. The genetic variation of immunity efficacy is well known [60, 153] but there is no clear understanding of this genetic cost of inducing an immune response in

invertebrates. The only study we know of, failed to observe it in *D.melanogaster* They found only physiological costs in some of the environment they tested [111]. We investigate the genetic cost of inducing immune responses by studying how induced immune costs are correlated with other life history traits, immune responses' efficacy and other immune costs [60, 106, 153], in different full-sib families of mosquitoes.

Immune responses overload

An alternative to explain the limits of immunity could be that the immune responses might be overloaded by the immune challenge and not only limited because of the physiological or genetic immune costs or the parasite immunosuppression. There could be some physiological limits to immune responses that are often overlooked but some examples exists, like in honeybees where the melanisation response is limited by the replenishment of phenoloxidase reserves [154]. We study this hypothesis, as a better understanding of such overloads could enlighten us about the resistance of mosquitoes to parasites.

Vector competence and the larval environment

Host-parasite interactions in different environments, have been largely studied in adult mosquitoes [122, 151]. However, food availability and temperature could also have an indirect impact on vector competence through their influence on larval development [155–157]. Variations in larval environmental factors have "carry on" effects on adult traits [158–161], as larval food influences adult lifespan [158, 162] and larval temperature has an effect on the transmission of malaria [161] and chikungunya [163]. Part of the indirect influence of the larval environment might just be linked to the influence of larval environmental factors on adult size [160, 164], as size is correlated with the probability of taking a blood meal [165] and the probability of infection [164]. Besides, mosquito size is also correlated with longevity [158, 166] and survival of infected mosquitoes [164]. We study the influence of larval environmental factors interactions on vector competence, while controlling for mosquito size.

Larval environment, mosquito size and survival

We were still puzzled by the effect of mosquito size and we decided to investigate more in detail the known relationship of this life history trait with longevity [158, 160, 162, 165–168]. Besides, a given mosquito size can be the result of different larval environments, as size is positively correlated with larval food [160, 162] and negatively correlated with larval temperature [164, 169]. We want to know if the influence of a given mosquito size on longevity, is different regarding the larval environment.

1.3 Research aims

We studied the ecological immunity of mosquitoes to improve the climate based epidemiological modelling of malaria transmission and the vector control. We investigated first the genetic cost of inducing immunity, the correlations between costs and the correlations between costs and efficacy (chapter 2). We then studied the potential overloading of the immune system to help in explaining the lack of resistance of some mosquitoes to malaria parasites in the wild (chapter 3). After that, we studied the consequences of the larval environment on vector competence (chapter 4). Finally we investigated the influence of larval environments on the relationship between adult size and longevity (chapter 5).

Chapter 2

2. Genetic correlations between cost and efficacy of mosquito immune responses

2.1 Abstract

Encapsulating parasites with melanin and killing them with antimicrobial peptides are two major immune responses of insects. How effective these pathways evolve to be depends on the costs and benefits of the immune responses, but also on the interactions between the pathways. We studied with a quantitative genetic approach three aspects of these interactions with the mosquito *Anopheles gambiae*: (i) whether the costs of inducing the two immune pathways have a genetic underpinning, (ii) whether there is a correlation between the costs of the two immune pathways and (iii) whether the cost of the melanisation response is correlated with its efficacy. Both immune responses were costly, leading to earlier death after the immune challenge, with little indication of genetic variation (i.e. of variation among mosquito families). However, there were unexpected negative correlations between the costs of the two immune pathways and between the cost and the efficacy of the melanisation response. Thus, the complex interactions between the benefits and costs of immune responses may concur to a less simplistic vision of the evolution of immunity with consequences on the underlying epidemiology of parasitic diseases.

2.2 Introduction

The melanisation response – the production and deposition of a dark layer of melanin encapsulating parasites – and the production of anti-microbial peptides that bind to and kill parasites are two major immune responses of insects [20, 63, 67, 76–78]. While our understanding of the mechanisms and genetic basis [76] of these pathways has increased considerably, we still do not know why

many individuals can clear invading parasites, while others, despite the obvious benefit of warding off disease, remain susceptible to infections.

The ideas of evolutionary ecology help to understand this variation. The general idea is that immunity is costly, with antagonistic pleiotropy [60, 108, 148, 152], so that stimulating immune functions decreases the fitness associated with other life history traits [67, 107–113]. This cost could be physiological or genetic.

There are physiological costs of inducing immune responses in mosquitoes [52] and an example is the shorter life span in immune challenged bumblebees under starvation [108]. Besides, there are genetic costs of maintaining an immune responses, as they are negative genetic correlation between immune traits and other life history traits [112]. Evolution will lead to a balance between the benefits (warding off disease) and the costs of immune responses [60, 153]. This idea implies that there is also a genetic variation of the costs of inducing immunity; there is however little evidence for such variation. The only study we know of, failed to observe it in *D.melanogaster* and only found physiological costs in some of the environment they tested [111].

In addition, the variation in a particular immune pathway may be a consequence of the correlations between its efficacy and that of other pathways, between the efficacy of the pathway and its cost, or between its cost and the costs of other pathways [60, 106, 153]. Indeed, immune pathways appear to be correlated in mosquitoes [115] and other insects [116, 117].

We investigated the genetic cost of inducing immune responses and such correlations within the immune system with the melanisation and the antimicrobial responses of the mosquito *Anopheles gambiae*. With a quantitative genetic approach we studied three questions. (i) Is there any genetic variation for the costs of inducing immunity? (ii) Are the costs of the two immune pathways genetically correlated? (iii) Are the cost and the efficacy of the melanisation response genetically correlated?

2.3 Methods

We assayed the genetic basis of the costs and their correlations by comparing full-sib families [170, 171]. To obtain enough mosquitoes and to reduce potential

maternal effects, we let the males and females of each full-sib family mate with each other and used their offspring, reared individually and in identical conditions, for all measurements.

The experiment was done with the Kisumu colony of *Anopheles gambiae s.s*. The mosquitoes were held in a climate chamber maintained at 26±1°C, 70±5% relative humidity and, a 12:12h light: dark cycle. To obtain the full-sib families, we haphazardly selected blood-fed females from the colony and let each of them lay eggs within a plastic cup. Each larva was reared in a well of a 12-well-plate containing 3ml of deionised water to which we added Tetramin™ baby fish food daily (day of hatching: 0.04 mg per larva; 1 day old: 0.06 mg; 2 days old: 0.08 mg; 3 days old: 0.16 mg; 4 days old: 0.32 mg, 5 days old or older: 0.6 mg). Fifty pupae of each family were placed into a cage. The mosquitoes were able to mate within full-sib families and the females were blood-fed three to four days after emergence; all adults had access to 10% sugar solution. The larvae of the second generation were reared like the first, except that each pupa was put into a cup with access to 10% sugar solution and only females were kept. From the 12 families with enough individuals to run the experiment, we chose eight haphazardly.

Inoculations

We inoculated female mosquitoes with either 0.1µl of liquid sterile Luria Bertani's (LB) broth or one of the following three treatments with the same volume of LB: heat-killed bacteria (*Escherichia coli*), control beads (glass beads), or immune-challenging beads (Sephadex beads). Over a period of six days we inoculated 45 mosquitoes per treatment every day; each mosquito was chosen haphazardly from one of the eight families. Thus, we inoculated a total of 1080 mosquitoes, 135 from each family.

To inoculate the mosquitoes, we chilled them briefly on ice and laid them on their right sides, and inoculated them in the haemolymph with a micro-capillary glass tube pushed through the left part of the thorax [102].

To induce melanisation, we inoculated female with one negatively charged Sephadex CM C-25 bead (Sigma-Aldrich, Steinheim, Germany) rehydrated in saline solution (1.3mM NaCl, 0.5mM KCl, 0.2mM CaCl2 [pH 6.8]) with 0.001%

methyl green to aid in visualization [102]. Glass-beads were used as a control, for they do not induce a melanisation response in *Anopheles gambiae*.

To induce the antimicrobial responses, we injected about 20'000 heat-killed *Escherichia coli* resistant to tetracycline and kanamycin. Bacteria were grown on LB broth Agar with 25 mg/L of tetracycline and 50 mg/L of kanamycin. Bacteria were then placed in liquid LB with the same concentrations of antibiotics and left to grow for 12 hours in an incubator at 37 C° and with 200 rpm. The bacteria were then concentrated to obtain 200'000 bacteria per µl. Finally, the bacteria were heat-killed in an autoclave at 110 C° during 25 minutes.

After being inoculated mosquitoes were given the opportunity to feed on cotton soaked in a 2 % sugar solution; this is five times less than the standard sugar level we use, so that it was more likely to detect any costs of immunity.

Costs and efficacy of immune responses

As cost of immunity, we assayed the time between inoculation and death. Survival was checked every 12 hours for the first ten days and then every 24 hours.

For mosquitoes inoculated with Sephadex beads, we took a digital image of each bead at a standard light setting with a microscope (Olympus® BX 50 equipped with a CC-12, Soft Imaging System), and used the software ImageJ (version 1.47f7) to measure the cross-sectional area of each bead and its mean grey value. The grey-value was standardized by linear interpolation to a value between 0 (which corresponded to the grey value of an unmelanised bead) and 100 (which corresponded to a heavily melanised bead).

To test for possible effects of size on the costs and efficacies of immune response, we fixed both wings onto slides and measured them from the tip to the distal end of the alula (excluding the fringe) with the software ImageJ; we used the mean length of the two wings in our analyses.

Analyses

All analyses were conducted in R 3.0.2. We tested the data for normality with Shapiro tests and for homoscedasticity of the variance with Bartlett tests. We removed from the analyses the 50 mosquitoes that we had inoculated with a

bead but in which we could not find it after dissection. As wing length had no significant effects, we present here the results without wing length.

Genetic variation of the costs of the immune responses

We analysed the costs of melanisation and of the antimicrobial response separately, considering for the former the mosquitoes inoculated with a Sephadex or a glass bead and for the latter those inoculated with dead bacteria or LB. We analysed the time between inoculation and death with a linear mixed model that included family, immune stimulation (bead type or bacteria vs. LB), age at inoculation and their interactions as independent variables. Family and its interactions were considered as random effects. Survival time was log-transformed to fit a Gaussian distribution in both analyses.

Correlations

As we could not measure the two costs of immunity and the efficacy of melanisation in the same individuals, we analysed the correlations using the mean values within families and day of inoculation. The cost of an immune response for a given family inoculated on a given day was defined as the difference between the mean survival times of the mosquitoes in that family inoculated on that day with the control (glass bead or LB) and those inoculated with the immune stimulus (Sephadex bead or dead bacteria). Note that this definition excludes the possibility that our measure of cost is confounded by the cost of wound healing.

We then analysed with an analysis of covariance the mean cost of the melanisation response as a function of the mean efficacy of melanisation (or the mean cost of the antimicrobial response), age at inoculation and their interaction as independent variables.

2.4 Results

Genetic variation of the costs of the immune responses

The time between inoculation and death (Fig.1a) was lower for mosquitoes inoculated with Sephadex beads (mean=6.8 days ± sd=9.27) than for their

controls (11.3 days ± 10.36) (c^2=42.0; P<0.001). It decreased with age at inoculation (c^2=85.7; P<0.001); the effect of age at inoculation was stronger for mosquitoes inoculated with Sephadex beads than for controls (interaction: c^2=15.0; P<0.001). However, there was no significant difference among families either in survival (5.80% of the total variance; c^2=0.01; P=0.903) or in the difference of survival between the two bead types, which ranged from -0.3 days to 7.8 days (c^2=2.7; P=0.437).

The overall pattern was similar for the cost of the antimicrobial response (Fig. 1b). The day of death after injection was lower for mosquitoes inoculated with heat-killed *E. coli* (10.5 days ± 9.90) than for their controls (13.6 days ± 11.54) (c^2=6.6; P=0.01), and it decreased with age at inoculation (c^2=48.3; P<0.001). However, there was no interaction between age at inoculation and the bacteria treatment (c^2=0.7; P=0.399). There was no significant difference among families either in survival (5.29% of the total variance; c^2=1.8; P=0.18) or in the difference of survival between the two bacteria treatments, which ranged from -1.9 to 9.0 days (c^2=0.6; P=0.902).

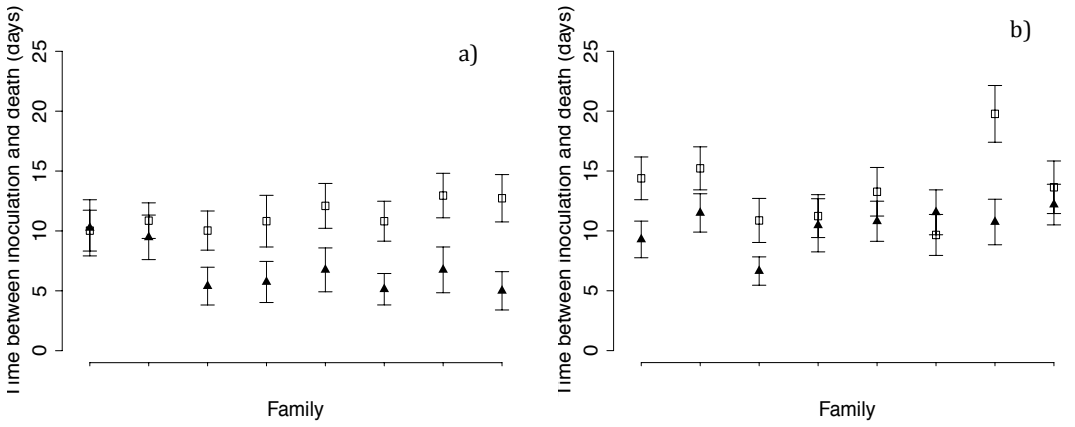

Fig.1 Mean number of days between inoculation and death of mosquitoes for each family. The solid triangles show the mean of the survival period within a family after immune-stimulation, the open squares show the mean within a family after inoculation with the control. The vertical lines represent the standard errors of the means. (a) Sephadex beads vs, glass beads. (b) Heat-killed bacteria vs LB broth. In both panels, the families are ordered by the cost of the melanisation response (i.e. the difference between the survival times of controls and of Sephadex bead-inoculated mosquitoes)

Trade-off between the two immune costs

The mean cost of melanisation (Fig.2) was negatively correlated with the mean cost of antimicrobial response ($F = 4.10$; $P = 0.01$). The day of inoculation tended to increase the cost of melanisation ($F = 2.23$; $P = 0.07$), but there was no indication of an interaction between the day of inoculation and the cost of antimicrobial response ($F = 0.67$; $P = 0.64$).

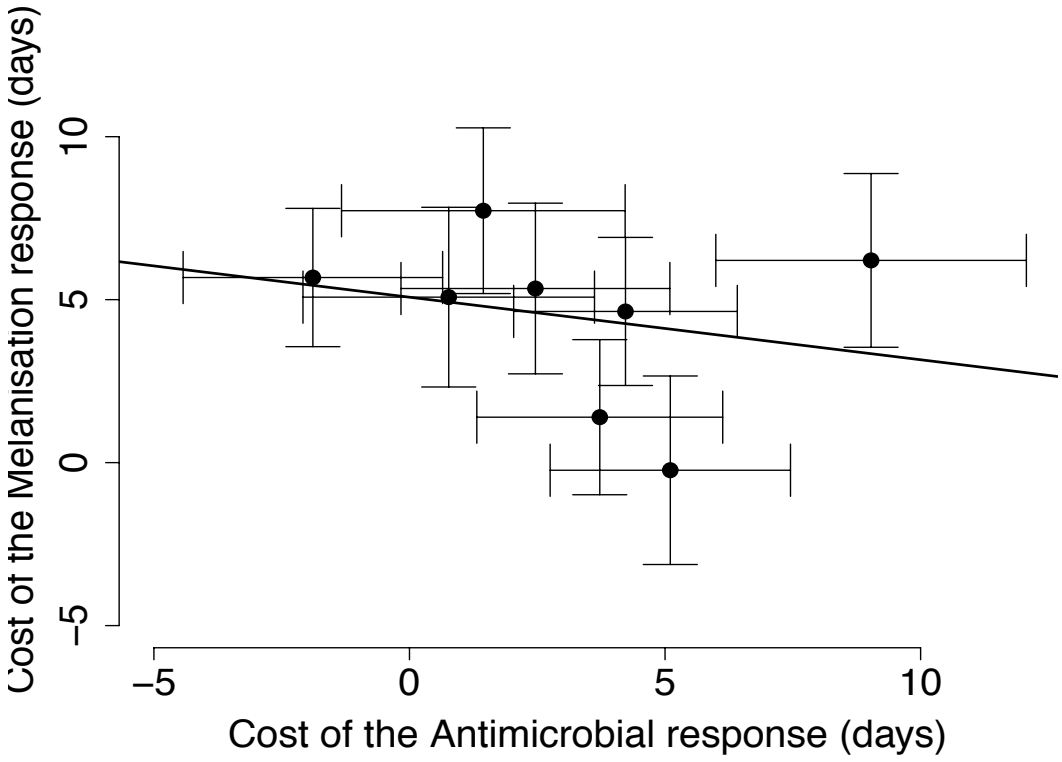

Fig.2 Trade-off between cost of the melanisation response and cost of the antimicrobial response. The cost of an immune response is represented by the difference of time from inoculation to death between mosquitoes inoculated with the control and those inoculated with the immune stimulation. Each symbol represents the mean value within a family. The horizontal and vertical lines represent the standard errors. Note that the analysis was done with the means within age at inoculation and family, while the figure pools the days of inoculation to show only the family-means (see Methods).

Correlation between the cost and the efficacy of melanisation

The mean cost of melanisation (Fig.3) was negatively correlated with the mean efficacy of melanisation ($F = 16.1$; $P < 0.001$). There was no influence of the day of inoculation ($F = 0.13$; $P = 0.71$) or of the interaction between efficacy and day of inoculation ($F = 1.32$; $P = 0.25$).

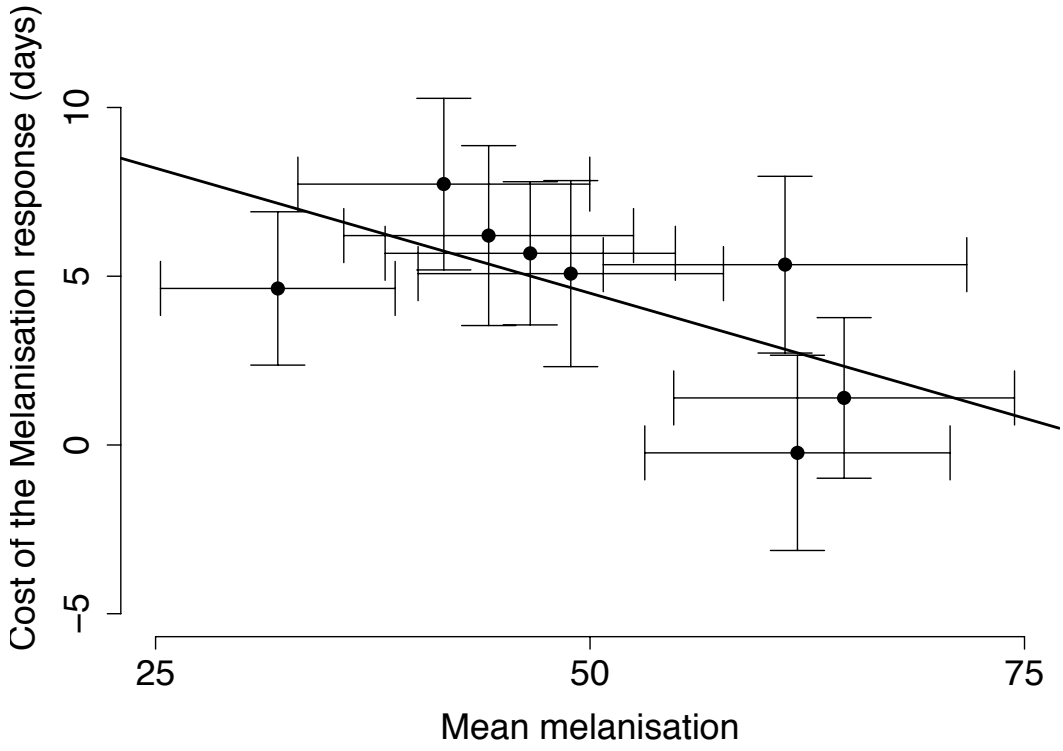

Fig.3 Association between cost of melanisation and melanisation efficacy. Melanisation is expressed as a value from 0 (for non-melanised beads) to 100 (for the most heavily melanised one); the cost of melanisation is represented by the difference of time from inoculation to death between mosquitoes inoculated with a glass bead and those inoculated with a Sephadex bead. Each symbol represents the mean value within a family. The horizontal and vertical lines represent the standard errors. Note that the analysis was done with the means within age at inoculation and family, while the figure pools the days of inoculation to show only the family-means (see Methods).

2.5 Discussion

We corroborated many other studies in which stimulating the immune response – here the melanisation and the antimicrobial responses – reduces the lifespan of insects [108]. While we could not detect any genetic variation (i.e. variation among full-sib families) of the costs of inducing either immune response, we observed a negative genetic correlation between the costs of the two immune responses. In other words, genotypes that easily mounted an immune response against one type of antigen, could only mount an immune response against another at a great cost. We also observed a negative genetic correlation (based on family means) between the cost of the melanisation response and the efficacy of the response. This may suggest that the cost of an immune response is less due to the production of the effectors than to the ease with which the immune response can be stimulated, as suggested by a study showing that neutral Sephadex beads are more strongly melanised but lead to a lower cost (with regard to fecundity) than the negatively charged Sephadex beads we used here [107].

A negative correlation between cost and efficacy – as the lifespan reduction was shorter for mosquitoes with a strong melanisation response – may be unexpected, but corroborates a study with aphids, where the most protective isolates of endosymbiotic bacteria, against parasitoid wasps, had the least fitness costs on aphids, with less reduction in lifespan and reproduction [118]. Such a negative correlation may depend on the immune pathway. Indeed, we found in an earlier study that the efficacy of the melanisation and the antibacterial responses [115] are positively correlated and here we observed a negative correlation between melanisation efficacy and cost. This implies that the cost and the efficacy of the antibacterial response are positively correlated, given the negative correlation between costs. Another study [116], however, measured a trade-off between the efficacies of the two immune responses, suggesting that the cost and the efficacy of the antibacterial response should then also be negatively correlated.

If such negative correlations are indeed frequent, they could have profound implications on our conception of the evolution of immune responses. A common idea is that the evolution will balance the costs and benefits of each immune

response, so that we would expect resources to be allocated to immune responses according to the parasite pressures [114]. Now, our first observation suggests that increased allocation towards one immune pathway would increase its efficacy and thereby decrease its cost. This would lead to a runaway selection for that immune response up to its maximal efficacy. In addition, our second observation suggests that the decreasing cost of this immune pathway would increase the cost of other pathways, making it more difficult to maintain their efficacy. Thus, while the general prediction of evolutionary immunology is that each immune response is at an intermediate efficacy, balanced by its costs, our observations could predict that the selection of one immune response prevents other immune pathways from being effective.

One caveat of this scenario is that evolution depends on more than just the longevity. The efficacy of an immune response could be positively correlated with the cost of this immune response with regard to reproductive success, as the melanisation response decreases fecundity [172], despite being negatively correlated with one of its components, i.e. longevity.

To conclude, given our observations, the idea that evolution will balance the benefits and costs of an immune response to obtain an optimal level of resistance against parasite infections may be too simplistic. The immune system is a complex network of interactions, not only in terms of benefits but also in terms of costs. In our study, we found two unexpected interactions as the cost and benefit of one immune response, and the costs of two immune responses were negatively correlated. Such correlations between immune traits and costs will shape the evolution of immunity in host parasite interactions with implications for vector control programs and epidemiological models.

Chapter 3

3. Overloading the immunity of the mosquito *Anopheles gambiae* with multiple immune challenges

3.1 Abstract

Melanisation – the production and deposition of a layer of melanin that encapsulates many pathogens, including bacteria, filarial nematodes and malaria parasites – is one of the main immune responses in mosquitoes. Can a high parasite load overload this immune response? If so, how is the melanisation response distributed among the individual parasites? We considered these questions with the mosquito *Anopheles gambiae* by inoculating individuals simultaneously with one, two or three negatively charged Sephadex beads, and estimating the melanisation as the darkness of the bead (which ranges from about 0 for unmelanised beads to 100 for the most melanised beads of our experiment). As the number of beads increased, the average degree to which beads were melanised decreased from 71 to 50. While the darkness of the least melanised bead in a mosquito decreased from an average of 71 to 35, the darkness of the most strongly melanised one did not change with the number of beads. As the number of beads increased, the mosquito's immune response became overloaded. The mosquito's response was to prioritise the melanisation of one bead rather than distributing its response over all beads. Such immune overloading may be an important factor underlying the evolution of resistance against vector-borne diseases.

3.2 Introduction

Immune responses are complex pathways that can kill invading pathogens and thus protect individuals from harmful infections. Many parasites have therefore evolved mechanisms to avoid the immune response by, for example, hiding within cells [100, 101], switching their surface antigens to prevent recognition [97–101], or actively suppressing the response [100, 102, 103].

Alternatively, the immune response may simply be overloaded by the immune challenge. In other words, if the infectious dose is high enough, the immune response might no longer cope with all of the parasites. Although this possibility appears to be generally overlooked, it finds support, for example, by experimental data suggesting that the immune melanisation response of honey bees is limited by their ability to replenish the phenoloxidase reserves needed for melanisation [154].

An overloaded immune system would be visible as an outcome lying between two extremes. At one extreme, few parasites could be targeted and dealt with optimally, while the remaining ones cannot be dealt with. At the other extreme, all parasites could be dealt with similar, but weak efficacy.

We considered these possibilities with the melanisation immune response of the mosquito *Anopheles gambiae* [76]. In mosquitoes, this immune response is effective against bacteria [173], filarial nematodes [123] and, in some cases, *Plasmodium* [63, 174].

A standard tool to study the melanisation response is to inject a small bead into the thorax and to measure the degree to which it is encapsulated with melanin [173]. While in many studies [63, 123, 174], only one bead is injected, we studied the potential immune overloading by investigating the degree to which a mosquito could melanise one, two or three beads injected into its thorax.

Beads are useful in this context, for our aim was to investigate the direct effect of immune stimulation on the immune response, without having to deal with the complicating effects of an invading pathogen such as pathogenicity or immune-suppression, which may be linked to pathogen load. Furthermore, we considered only the melanisation response, for it is difficult to evaluate the efficacy of the immune system against dead bacteria (and, again, we did not wish to consider a living pathogen like Lambrechts et al. [115]).

3.3 Methods

The experiment was performed at 26±1°C, 70±5% relative humidity and a 12:12h light: dark cycle. We used the Kisumu laboratory strain of *An. gambiae* originating from western Kenya [175]. We selected newly hatched larvae

haphazardly and reared them individually in 12-well-plates filled with 3ml of deionised water to which we added Tetramin™ baby fish food daily (day of hatching: 0.04 mg per larva; 1 day old: 0.06 mg; 2 days old: 0.08 mg; 3 days old: 0.16 mg; 4 days old: 0.32 mg, 5 days old or older: 0.6 mg) [176]. Each pupa was put into a 180 ml plastic cup covered with mosquito netting. After emergence, males were removed and females were given access to 10% sugar solution.

Two days after emergence, 60 females were chilled on ice. We inoculated each female with 1, 2 or 3 negatively charged Sephadex CM C-25 beads (40–120 μm in diameter, Sigma-Aldrich, Steinheim, Germany), injected simultaneously together with 0.1 μl sterile saline solution (1.3 mM NaCl, 0.5 mM KCl, 0.2 mM CaCl2 [pH 6.8]) into the left side of the thoracic cavity [102, 177]. We added 0.001 % methyl green to the saline to help us see the transparent beads. After inoculation, mosquitoes were returned to their cups and given 10 % sugar solution.

Two days later, i.e. 24 hours after the melanisation of a single bead has reached its plateau [174], we dissected the mosquitoes that were alive to measure their wing length (as a proxy of body size), the size of the beads and the degree of their melanisation. The mosquitoe's wings were removed, fixed onto slides and measured from the tip to the distal end of the alula (excluding the fringe) with the software Image J (version 1.47f7). We used the mean length of the two wings in our analyses. On a glass microscope slide we then separated the thorax from the head and from the abdomen using forceps in the same saline solution used for injection. We opened the thorax with forceps, retrieved the beads and put them onto a slide in solution to take the picture.

In this, and previous experiments, the beads were found in the thorax and had not moved to the abdomen. When we found several beads, they were not in contact with each other. Most beads were floating freely in the haemocoel, but some had tissues attached. In this and other experiments, we did not find any effect of the presence of tissue to bead melanisation. We took a digital image of each bead at a standard lighting setting with a microscope (Olympus® BX 50 equipped with a CC-12, Soft Imaging System), and measured the cross sectional area of each bead and its mean grey value with the software Image J. The grey-value was standardized by linear interpolation to a value between 0 (which

corresponded to the grey value of an unmelanised bead) and 100 (corresponding to a heavily melanised bead).

We tested the data for normality with Shapiro tests and for homoscedasticity of the variance with Bartlett tests. As initial analyses of full models with interactions terms and models without non-significant interactions showed that the size of beads had no influence on the conclusions, we omitted bead size from the analyses shown here.

To assay whether the number of beads influenced the melanisation response, we analysed the melanisation of each bead with a linear mixed model (function lmer) that included the number of injected beads and wing length as independent variables and the mosquito as a random factor. In a second analysis, we assayed the variability of the melanisation response as a function of the number of beads. As there is no variability in mosquitoes inoculated with one bead, we could not perform a standard assay of repeatability. Therefore we analysed the bead with the highest melanisation or the lowest melanisation in each mosquito with an ANCOVA, again including the number of injected beads and wing length as independent variables. We analysed the survival rate two days after injection with a binomial GLM including the number of beads inoculated and wing length as independent variables. All analyses were performed with R version 3.0.2.

3.4 Results

Of the 60 mosquitoes we had inoculated, 44 were analysed (18, 12 and 14 mosquitoes inoculated with 1 bead, 2 beads or 3 beads, respectively). This difference in survival was statistically not significant (Chi-square=5.21, P=0.076).

The mean level of melanisation decreased from 71 (± sd=14) in mosquitoes inoculated with one bead, to 50 (± sd=27) in those inoculated with three beads (Fig. 1; Chi-square =8.47, P=0.01). There was no effect of wing length (Chi-square =0.106; P=0.74) or the interaction of number of beads and wing length (Chi-square =1.267; P=0.53).

While the highest melanisation per mosquito was independent of the number of beads (69 ± sd=19; F =0.380, P=0.686); the lowest melanisation decreased from 71 (± sd=14) in mosquitoes inoculated with one bead, to 35 (± sd=25) in mosquitoes inoculated with three (Fig. 1; F=12.76; P<0.001). Neither highest (F=0.376, P=0.543) nor lowest (F=0.13, P=0.72) melanisation were affected by wing length.

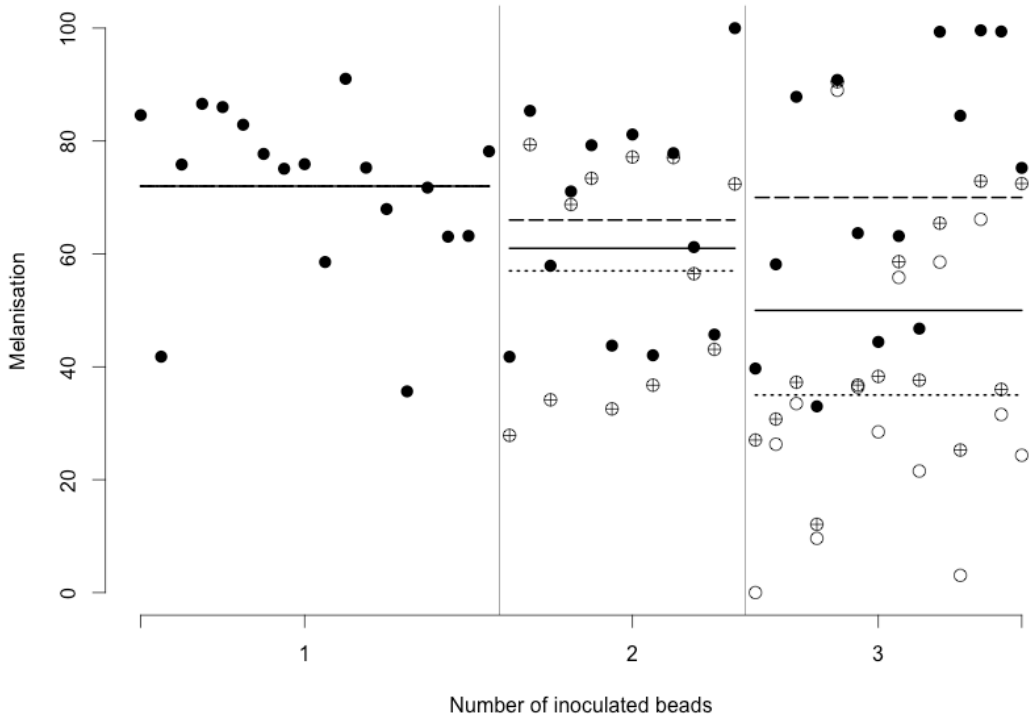

Fig.1. Bead melanisation as a function of the number of inoculated beads. Each point shows the melanisation of a single bead (with values ranging from 0 for non-melanised beads to 100 for the heavily melanised ones). The solid points represent the highest melanisation value per mosquito, the crossed points represent the intermediate melanisation value and the empty points represent the lowest melanisation value. The solid lines represent the mean melanisation per bead treatment, the dashed lines the mean of the highest melanisation and the dotted lines the mean of the lowest melanisation.

3.5 Discussion

Our data suggest that the mosquito's melanisation immune response becomes overloaded by a small number of injected Sephadex beads. When the immune response is overloaded, the strength of melanisation is not distributed uniformly among the beads, but that one bead is prioritised to the detriment of the immune response against others. Indeed, almost all mosquitoes were able to melanise one bead to a high degree, whether it had been inoculated with one, two, or three beads, but additional beads were melanised much less. The least-melanised bead in each mosquito inoculated with three beads was melanised only about half as much as the best one.

There are several mechanisms that could potentially lead to our results. Perhaps the most likely is simply due to the need to replenish phenoloxidase reserves [154]. Thus any of the rates in the complex pathway from recognition to melanisation [178] may constrain the production of melanin to the degree that not all beads can be completely melanised. In addition to a constraint on the degree of equilibrium, one might also expect that this constraint may lower the rate of melanisation. Thus, if we had waited longer, the degree of melanisation among beads within mosquitoes would have been less variable. However, melanisation generally takes place within hours [85] and reaches a plateau way within 24h in mosquitoes inoculated with only one bead [174]. It thus seems highly unlikely that a delay would influence the melanisation observed 48 hours after inoculation.

Mechanisms leading to preferentially targeting some beads over others are less clear. One might suspect that the degree of melanisation within a single mosquito is related to the characteristics of the bead, for example its size. However, we controlled for the size of beads in initial analyses, and it had no influence on the degree of melanisation. One could postulate a positive feedback underlying the regulation of melanisation. Then, the beads that initially (by chance) stimulate a slightly greater response will continue to stimulate the immune response more strongly and reach a greater level of melanisation. However, mechanisms for such a feedback are not known, and indeed, postulated feedback loops are negative [179, 180].

Whatever the mechanism, the fact that the immune response can be overloaded in a way that leads to variability of its outcome within individuals may have considerable implications for the transmission of infectious diseases. Mosquitoes, for example, clear most of the malaria parasites that infect them [181], but, in natural populations, many mosquitoes are unable to clear all of them, and the remaining few are enough to enable effective transmission. The reason for this is unclear. Whereas this ability to clear all parasites is often considered as a measure of qualitative resistance by mosquitoes with an intact immune response, the lack of complete resistance may reflect overloading of mosquitoes with otherwise effective immune responses.

The importance of this variability in the melanisation response could vary according to the type of parasite and some uncertainties remain, some parasites may be killed by the encapsulation, others may be killed by the oxidative cytotoxic compounds produced[80, 182]. So a lower melanisation response may not be sufficient to kill all pathogens. Nevertheless, we certainly expect that the degree of melanisation is essential for 'large' parasites such as filarial worms or malaria oocysts.

To conclude, the efficacy of the mosquito's melanisation immune response strongly decreased with the antigen load that stimulates it. Understanding the possible constraints on mosquito immunity by overloading the system, be it with regard to the melanisation response or other immune pathways, may help to understand the evolution of resistance and the transmission of disease.

See published version in Parasites and Vectors 2016.

Chapter 4

4. Larval environment influences the vector competence of the mosquito *Anopheles gambiae* for malaria

4.1 Abstract

While environmental factors such as temperature can influence the vector competence of mosquitoes directly, for example by affecting the longevity of the mosquito and the development of its parasite they may also have an indirect impact on the parasite's transmission. By influencing larval development, they may affect the adult traits that are important for the parasite's development and transmission. We studied the influence of two larval environmental factors, food availability and temperature, on the probability that *Anopheles gambiae* mosquitoes infected with the malaria parasite *Plasmodium berghei* survived to harbour sporozoites (the infectious stage of the parasite) in their salivary glands. This measure of vector competence dropped by about a third if we fed larvae half of our standard food regime. The effect of temperature during the larval period depended strongly on the food regime. At low food, increasing temperature from 21ºC to 29ºC increased vector competence from about 0.18 to 0.48, whereas at standard food, vector competence dropped from about 0.67 at 21ºC to 0.56 at 29ºC. Thus, ideas and models about the role of environmental change on the transmission of malaria (and other vector-borne diseases) must include how the environment changes adult life-history by influencing larval development.

4.2 Introduction

The competence of a vector to transmit an infectious disease is the result of a complex interplay between the parasite's and the vector's traits, and how the environment influences them. Rising temperature, for example, is expected to enable the parasite to develop more rapidly [131–133], but may decrease its chance of surviving its developmental period [131, 134], and it can shortens the vector's life-span [127]. Depending on the details of the interactions between

these traits, increasing temperature can, overall, increase or decrease vector competence [122, 135, 138, 139, 141, 142]. Nutrition also greatly influences vector competence [151] by affecting the infection load [130], the immune response [123] and the longevity [128, 129] of the vector.

In addition to such direct effects, the environment may influence vector competence indirectly by affecting larval development [155–157], thus having carry-on effect on the adult traits underlying vector competence [159, 160]. Food and temperature during larval development, for example, influence the longevity of adult mosquitoes [158], and larval temperature can influence the transmission of malaria [161] and chikungunya [163]. Part of this indirect effect may be a simple consequence of size: larval food [160] and temperature [164] influence adult size, which in turn influences the probability of infection [164], the longevity of mosquitoes [158, 166] and the survival of infected mosquitoes [164].

To understand better the complex interactions between the larval environment, the larval development, adult size and vector competence, we studied the combined effect of temperature and food during larval development on the probability that the mosquito *Anopheles gambiae* survives infection by the malaria parasite *Plasmodium berghei* and harbours sporozoites in its salivary glands.

4.3 Methods

We used the Kisumu strain of *An. gambiae* [175]. Newly hatched larvae were placed individually in 12-well-plates containing 3ml of deionised water, to which we added Tetramin™ baby fish food daily. The mosquitoes were reared at 21ºC, 25ºC or 29ºC, and fed either a standard larval diet or half of the standard. The standard diet at 25ºC and 29ºC was 0.04 mg per larva on the day of hatching, 0.06 mg for 1 day old larvae, 0.08 mg for 2 day olds, 0.16 mg for 3 day olds, 0.32 mg for 4 day olds, and 0.6 mg for 5 day olds and older larvae. At 21ºC pupation is about 3 days later in our lab than at the higher temperatures (unpublished data). We reduced daily standard food at this temperature to achieve about the same total amount of food during larval development (day of hatching: 0.04 mg per

larva, 1 day old: 0.05 mg, 2 days old: 0.06 mg, 3 days old: 0.08 mg, 4 days old: 0.12 mg, 5 days old: 0.19 mg, 6 days old: 0.32 mg, 7 days old: 0.38 mg, 8 days old: 0.45 mg; 9 days old and older: 0.45 mg). Each pupa was put into a 180 ml plastic cup covered with mosquito netting. After emergence, males were discarded and females were given access to 10% sugar solution at 25±1ºC. (Thus the adult environment was identical for all mosquitoes).

Infection

The sugar was removed from the cups 24 hours before the infectious blood-meal (4 to 5 day old mosquitoes). For each food and temperature treatment the mosquitoes were grouped into four cups (with close to equal numbers per cup), which were randomly allocated to four mice harbouring the infectious gametocytic stage of *Plasmodium berghei* ANKA transformed with Green Fluorescent Protein (GFP, obtained from the Institute of Cell Biology, University of Bern, Switzerland). The mice were anaesthetized by intra-peritoneal injection of 8.5mL/kg of a mix of Xylazine Xylasol® (solution: 20mg/mL), Ketamine Ketasol ® (solution: 100mg/mL) and PBS (Phosphate buffered saline), and they were placed onto the cups containing the mosquitoes for 10 minutes. One day after the blood meal, female mosquitoes that were fully fed (292 out of 402) were put individually in cups with 10% sugar solution and kept at 19±1ºC (Higher temperatures block the development of the parasite).

Dissection

Survival was assessed every 24 hours up to 21 days after infection, when all surviving mosquitoes (185 mosquitoes) were dissected in LOCKE solution [183]. The wings were measured from the tip to the distal end of the alula (excluding the fringe) [172] with the software Image J (version 1.47f7); we used the mean length of the two wings for our analyses. We checked the salivary glands for the sporozoites with a fluorescent microscope sensitive to GFP.

Analyses

Wing length was analysed with an ANOVA including larval temperature, larval food and their interaction. All other analyses were binomial GLMs. Each one

included larval temperature, larval food and their interaction and wing length as a covariate. We analysed three outcomes: survival up to the time of dissection, infection success (i.e. whether we found sporozoites in the salivary glands, considering only the mosquitoes that had survived up to dissection), and "vector competence" (mosquitoes were classified as competent if they survived until dissection and harboured sporozoites in their salivary glands). All analyses were performed with R 3.0.2.

4.4 Results

Wing length decreased from 3.33mm (±se 0.002) in mosquitoes that had been reared at 21ºC to 3.21mm (± 0.002) at 25ºC and 2.99mm (± 0.002) at 29ºC, ($F=99.55$; $P<0.001$). Wings were longer, if larvae had obtained the standard diet (3.26mm ± 0.001) than low food (2.99mm ± 0.001), ($F=143.52$; $P<0.001$). There was no interaction between larval temperature and food ($F=0.40$; $P=0.672$).

Mosquitoes were more likely to survive for 21 days after infection, if they had been fed a standard diet as larvae (0.68; 95% confidence interval 0.61-0.75) than if they had obtained half the amount of food (0.55; 0.45-0.64), and larger mosquitoes had a greater chance of survival (Table.1), with survival increasing by 0.05 per 0.1mm wing length. Neither temperature nor the interaction between food and temperature significantly affected survival (Table 1).

Surviving mosquitoes were more likely to harbour sporozoites, if they had obtained the standard diet (0.90; 0.84-0.95) than the low diet (0.73; 0.60-0.82). In contrast to its effect on survival, wing length had no significant effect on the sporozoite rate. Although there was no direct impact of temperature during larval development on sporozoite rate, temperature affected the impact of food (Table 1). The difference in sporozoite rate between the standard and the low diet decreased from 0.64 (0.31-0.85) at 21ºC, to 0.14 (-0.01-0.33) at 25ºC and 0.04 -(0.14-0.23) at 29ºC.

Vector competence (the combination of survival up to dissection and the likelihood of harbouring sporozoites) was higher if larvae had been reared on the standard diet (0.63; 0.56-0.70) than on low food (0.41; 0.32-0.50) and when the mosquitoes were larger (Table.1) with vector competence increasing by 0.05

per 0.1mm wing length. The effect of food was strongly affected by larval temperature (Table 1). After a standard diet, vector competence tended to decrease with increasing temperature from 0.67 (0.52-0.79) at 21ºC, to 0.56 (0.43-0.69) at 29ºC, but after a low diet vector competence increased from 0.18 (0.06-0.43) at 21ºC to 0.42 (0.28-0.56) at 25ºC and 0.48 (0.34-0.62) at 29ºC (Fig.1).

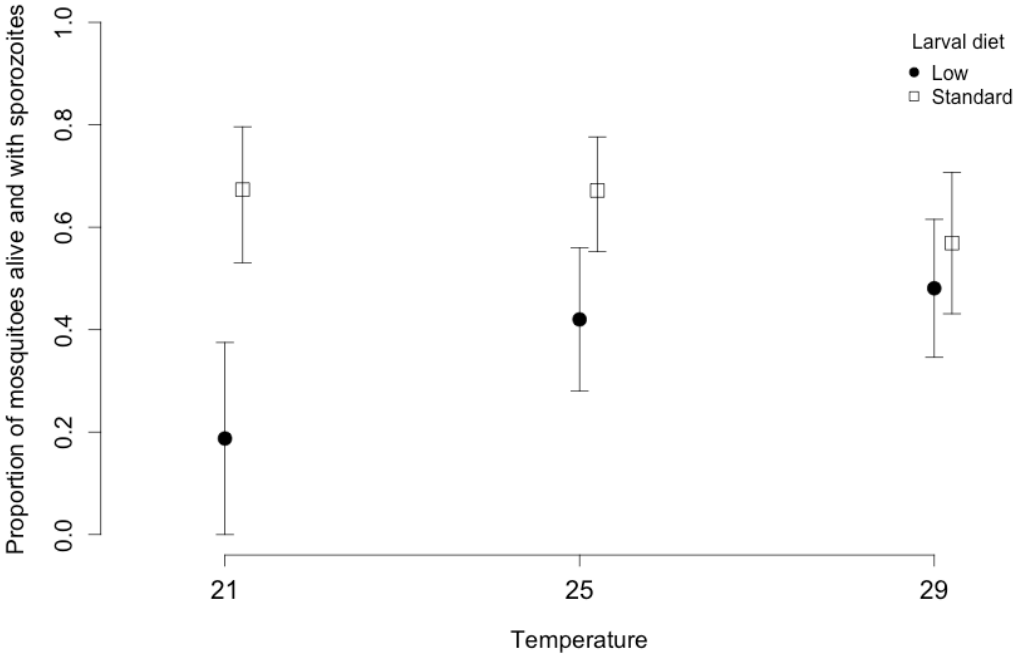

Fig.1 Proportions of mosquitoes that survived for 21 days after infection and had sporozoites in their salivary glands, as a function of larval temperature and diet. The solid points represent the low food larval diet and the empty squares the standard food larval diet. The vertical lines represent the confidence intervals of the proportions.

		Survival		Infection		Vector competence	
Factor	df	c^2	P	c^2	P	c^2	P
Temperature	2	0.14	0.92	1.18	0.55	0.87	0.64
Food	1	5.16	0.02	10.94	<0.001	13.83	<0.001
Temperature*Food	2	0.56	0.75	10.82	0.004	7.19	0.02
Wing length	1	19.17	<0.001	0.00	0.97	11.15	<0.001

Table.1 Summary of statistical analyses

4.5 Discussion

Two aspects of larval environment – food and temperature – interacted to determine the vector competence of *A. gambiae* for malaria. Low food decreased competence, but only at low temperature; at high temperature, food had limited effect. These results complement an earlier study [161] showing that undernourishment of mosquitoes during larval development decreases the oocyst load (but not the prevalence) of malaria. They also show, however, that such results must be taken with caution, as the effect of one environmental factor can be influenced by other environmental factors.

In our experiment the effect on vector competence was due to a combination of survival during the parasite's development and the proportion of the survivors that harboured sporozoites. The larval environment influenced both traits. Part of these effects was simply due to the mosquito's size. Undernourishment and high temperature both led to smaller adults (as generally observed in invertebrates [184]) and smaller mosquitoes were overall less competent, confirming earlier studies [163, 185]. However, we found considerable effects of larval food and temperature after having controlled for the effects of size.

First, lower food decreased the parasite's and the host's survival. This may in part be due to the resources stored during the larval development [162] that can then be used, for example, to increase survival as an adult**.** Although lower levels of food generally increase the life-span of healthy individuals [186–188], it reduced the survival of infected mosquitoes through the parasite's development. This suggests that the resources acquired during larval development are critical to maintain an effective immune response and resistance against the parasite

[121, 124, 189]. On the other hand, the resources do not appear to help control the parasite's growth. Rather, the lower parasite's survival of undernourished mosquitoes in our study and in [17, 161] suggests that these mosquitoes do not have acquired enough resources to support the parasite's growth [162].
Second, the effect of temperature depended on the level of larval food and on the trait that was investigated. Temperature of adults clearly affects the survival of mosquitoes and the developmental rate of parasites, and thus the vectorial capacity [131, 151]. Larval temperature affects the size of the adults [159], which affects survival and perhaps parasite development. However, once we controlled for this indirect effect in our analysis, temperature had no effect on the survival of the mosquito. In contrast, low larval temperature impeded the parasite's development in the adult, but only if larval food had been low. At 25°C and 29°C the influence of food was smaller, perhaps because the faster development of the mosquitoes at higher temperatures gave less time for the difference in food to affect the storage of resources. These results suggest that the mosquito's ability to fight the parasite is weakest when the effects of temperature and food have conflicting effects on body size – low temperature increases adult size, but low food decreases it – and resources.

In conclusion, the larval environment influenced the vector competence for malaria of adult mosquitoes in a complex way. Thus, ideas and models about the role of environmental change on the transmission of malaria (and other vector-borne diseases) must include how the environment indirectly changes adult life-history by influencing larval development. In particular, we must consider the larval ecology to improve climate-based epidemiological modelling of malaria.

Chapter 5

5. The relationship between size and longevity depends on the larval environment in *Anopheles gambiae*

5.1 Abstract

Longevity, a key life-history trait that determines a mosquito's evolutionary fitness, is often associated with body size. Both traits are strongly influenced by the environment during the mosquito's development. Does the larval environment change the relationship between size and longevity? We considered this question using the mosquito *Anopheles gambiae* reared at three temperatures (21°C, 25°C, 29°C) and at two food levels (our standard food level and half of it). Both traits responded to the environment but the overall relation between size and longevity was weak. However, the slope of the regression of longevity on body size was affected by the combination of the two environmental factors. It was about null at 25°C, but the slope was negative at 29°C – standard food and at 21°C – low food, and it was positive at 29°C – low food and 21°C – standard food. Thus, the association between size and longevity is environment dependent.

5.2 Introduction

Longevity is a key life-history trait to determine a mosquito's evolutionary fitness [146, 148] and longevity is often associated with body size [158, 160, 162, 165–168]. Many environmental factors during larval development – e.g. food availability, competition with other larvae, temperature – affect the mosquito size [160, 164, 168, 169, 190] and also other life history traits, like age at pupation [168]. However, the effect on size may be different than the effect on other traits, as for example both lower food and lower temperature slow the larval development but have opposite effects on size [159, 160]. We generally expect lower food to increase longevity and lower larval temperature to increase longevity [158]. We may therefore expect larval environmental factors to modify

the effect of size on longevity. To study the influence of larval environmental factors on this relationship, we reared *A. gambiae* mosquitoes at three different larval temperatures 21°C, 25°C and 29°C, with a standard diet or half of it. We let the adult females blood feed and we measured their size and their longevity.

5.3 Methods

We used the Kisumu strain of *An. gambiae* [175]. Newly hatched larvae were placed individually in 12-well-plates containing 3ml of deionised water, to which we added Tetramin™ baby fish food daily. The mosquitoes were reared at 21ºC, 25ºC or 29ºC, and fed either a standard larval diet or half of the standard. The standard diet at 25ºC and 29ºC was 0.04 mg per larva on the day of hatching, 0.06 mg for 1 day old larvae, 0.08 mg for 2 day olds, 0.16 mg for 3 day olds, 0.32 mg for 4 day olds, and 0.6 mg for 5 day olds and older larvae. At 21ºC pupation is about 3 days later in our lab than at the higher temperatures (unpublished data). We reduced daily standard food at this temperature to achieve about the same total amount of food during larval development (day of hatching: 0.04 mg per larva, 1 day old: 0.05 mg, 2 days old: 0.06 mg, 3 days old: 0.08 mg, 4 days old: 0.12 mg, 5 days old: 0.19 mg, 6 days old: 0.32 mg, 7 days old: 0.38 mg, 8 days old: 0.45 mg; 9 days old and older: 0.45 mg). Each pupa was put into a 180 ml plastic cup covered with mosquito netting. After emergence, males were discarded and females were given access to 10% sugar solution at 25±1ºC. (Thus the adult environment was identical for all mosquitoes).

Blood meal

The sugar was removed from the cups 24 hours before the blood meal (4 to 5 days old mosquitoes). For each food and temperature treatment the mosquitoes were grouped into two cups (with close to equal numbers per cup), which were randomly allocated to four mice (obtained from the Institute of Cell Biology, University of Bern, Switzerland). The mice were anaesthetized by intra-peritoneal injection of 8.5mL/kg of a mix of Xylazine Xylasol® (solution: 20mg/mL), Ketamine Ketasol ® (solution: 100mg/mL) and PBS (Phosphate buffered saline), and they were placed onto the cups containing the mosquitoes

for 10 minutes. One day after the blood meal, female mosquitoes that were fully fed (187 out of 303) were put individually in cups with 10% sugar solution and kept at 19±1ºC (Higher temperatures block the development of the parasite).

Longevity

Longevity was the time between the blood meal and death. Survival was assessed every 24 hours upon death. The wings were measured from the tip to the distal end of the alula (excluding the fringe) [172] with the software Image J (version 1.47f7); we used the mean length of the two wings in our analyses.

Analyses

We did a regression analysis of longevity regarding wing length at each treatment. We did also an ANOVA of wing length regarding temperature, food and their interaction. Then, we did a path analysis, which estimated the parameters of the equation:

longevity = A + B temperature + C food + D wing length

where wing length is also influenced by larval temperature and food,

wing length = $\alpha + \beta$ temperature + γ food

The parameters of these equations were estimated with the function "plspm" in R 3.0.2. We estimated the precision of the model using a bootstrapping approach.

5.4 Results

There was no relation between longevity and size when putting all the larval treatments together (Figure.1.). When looking at longevity regarding size in the different environments (Figure.2.), there was no relation at 25°C whatever the diet (R^2=0.00 at standard food and R^2=0.00 at low food). However, the trend was negative at 29°C – standard food (R^2=0.04; Figure 2.a.) and at 21°C – low food (R^2=0.11; Figure 2.b.). It was positive at 29°C – low food (R^2=0.05; Figure 2.a.) and at 21°C – standard food (R^2=0.10; Figure 2.b.)

Mosquito longevity was smaller with standard food 28.07 days (±sd 12.0) than low food, 30.02 days (± 11.6). Longevity was smaller at 21°C, 27.5 days (± 13.2), than at 25°C, 29.1 days (± 12.6), and 29°C, 29.4 days (± 10.4). Longevity was

about the same, whatever the diet at 25°C. However, the longevity was higher with low food (30.3 days ± 9.22) instead of standard food (26.4 days ± 14.4) at 21°C and at 29°C (30.6 days ± 9.3; 28.3 days ± 11.2).

Mosquitoes were smaller with low food 3.01mm (±sd 0.02) than with standard food 3.24mm (± 0.01), ($F=90.14$; $P<0.001$). Mosquitoes were smaller at 29°C, 3.01mm (± 0.02), than at 25°C, 3.23mm (± 0.02), and 21°C, 3.27mm (± 0.02), ($F=35.63$; $P<0.001$). There was no significant interaction of food and temperature on mosquito size ($F=0.71$; $P=0.49$). Although, on one hand mosquitoes reared at 29°C – standard food and at 21°C – low food, had about the same mean size, 3.10 mm (± se= 0.01) and 3.09mm (± 0.01) respectively. On the other hand, mosquitoes reared at 29°C – low food and 21°C – standard food, had a different mean size, 2.92 mm (± se= 0.01) and 3.34mm (± 0.01) respectively. Mosquitoes reared at 25°C were smaller with low food 3.12 mm (± se= 0.03) than standard food 3.28 mm (± se= 0.03).

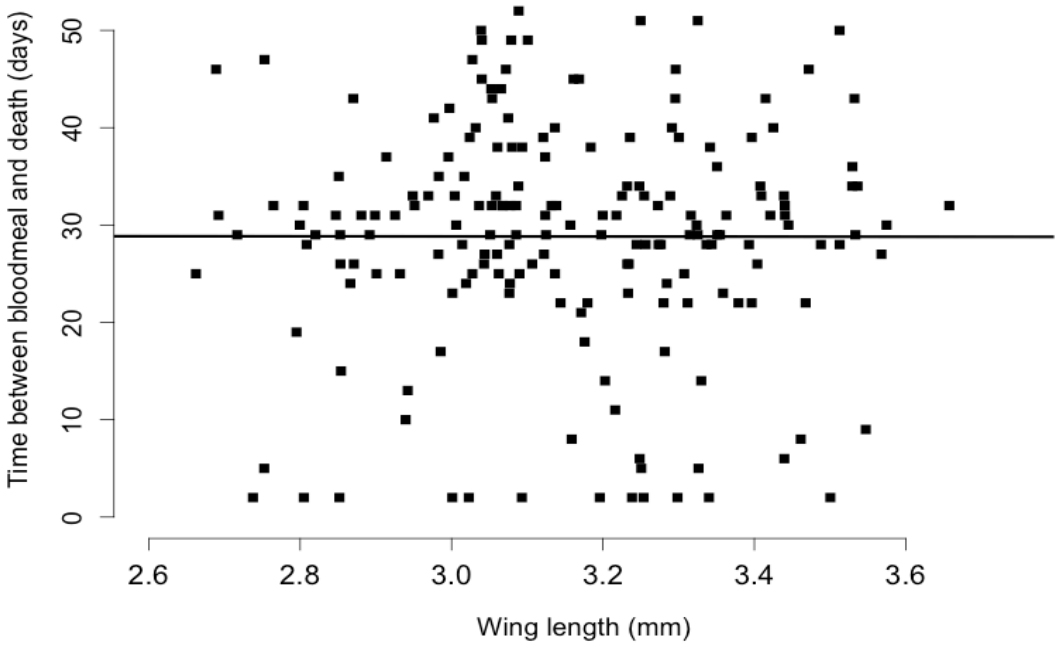

Figure.1. Longevity regarding mosquito size. Each point shows the time between the blood meal and death for each mosquito. The solid line represents longevity regarding wing length.

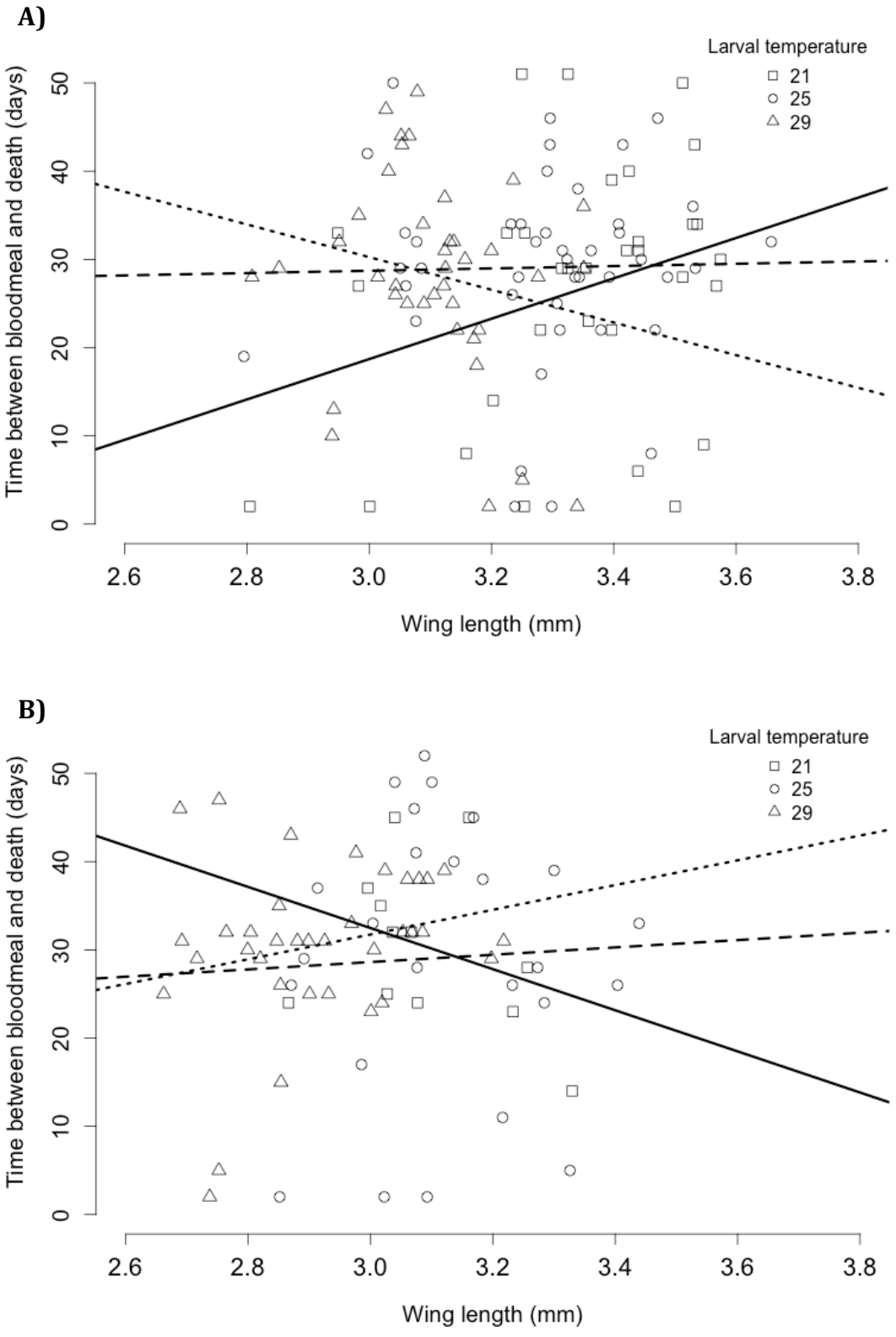

Figure.2. Longevity regarding mosquito size and larval temperature. Each point shows the time between the blood meal and death for each mosquito. A) At Standard food, B) At Low food. Squares are mosquitoes reared as larvae at 21°C, points at 25°C and triangles at 29°C. The solid line represents longevity regarding wing length at 21°C, the dashed line at 25°C, and the dotted at 29°C.

Looking at the path analysis, wing length had a positive direct relationship with longevity. Temperature had a positive direct effect on longevity but a negative indirect effect on longevity through wing length. Food had a negative direct effect on longevity but a positive indirect effect through wing length (Figure.3. and Table.1). Wing length had a positive relation with food but a negative one with temperature.

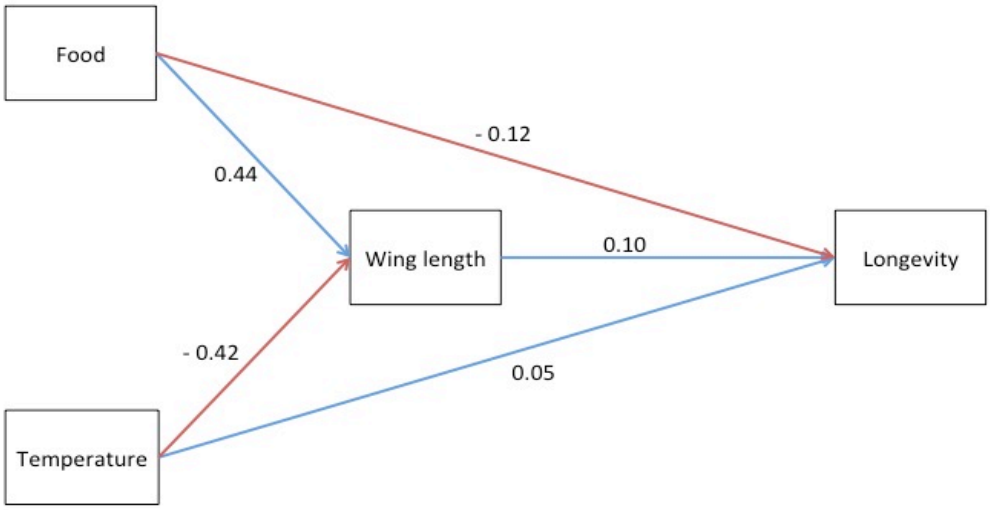

Figure.3. Path analysis for longevity regarding wing length, food and temperature. This is the model of the interactions between the variables to explain longevity. Red arrows represent negative relationships and blue arrows positive ones. The strength of each relation is figured on the arrow.

	Effect
food -> longevity	-0.12 (-0.27; 0.06)
temperature -> longevity	0.09 (-0.06; 0.24)
wing length -> longevity	0.10 (-0.10; 0.24)
food -> wing length	0.44 (0.33; 0.53)
temperature -> wing length	-0.42 (-0.50; -0.34)

Table.1 Coefficients in the path analysis. The direct effects of each variable on longevity and on wing length with the 95% confidence interval.

5.5 Discussion

Overall, the relationship between mosquito size and longevity was weak, even if we observed a positive effect of wing length on longevity. It was particularly weak at 25°C, as mosquitoes were larger with standard food but it had no influence on longevity. However, on one hand, the relation between size and longevity was negative at 29°C – standard food and at 21°C – low food, and on the other hand, it was positive at 29°C – low food and at 21°C – standard food.

First, this could be explained by a conflicting interaction between food and temperature during the larval development in the former and a synergic effect in the later, as mosquito size was positively correlated with food and negatively with temperature [158, 160, 164, 169]. Besides, the same mean mosquito size was obtained at 29°C – standard food and at 21°C – low food, meaning that the most stressful factor may dominate in each case to influence size. Growing large in such environment may then have negative consequences for the longevity of mosquitoes, as it may be more costly.

Secondly, we found a similar negative trend between size and longevity with increasing temperatures from 21°C to 25°C, at standard food, than Christiansen et al. [158] were smaller mosquitoes survived less when the temperature increases from 23°C to 35°C. However, here at low food the correlation was the opposite. When considering environmental effects, the interactions with other factors are key to a better understanding.

Longevity was negatively influenced by food and positively influenced by temperature in our study but it was more complicated when looking at the indirect effect of the interactions between food and temperature on size. The effect of size on fitness may vary regarding the larval environment but also regarding the life history traits considered – eg. reproductive success, immunity or the resistance to parasites. Indeed, smaller *Aedes aegypti* mosquitoes may be more resistant to dengue virus [185] or less resistant [191] depending on the studies. In both studies, size was manipulated by modifying mosquito density [185] but in the second the effect of competition was added [191].

To conclude, the relation between size and longevity will vary regarding the interactions between environmental factors. These interactions may constrain

mosquito development with carry on effects on adult size and repercussions on mosquito fitness. A better understanding of this relation could help modelling the transmission of vector borne disease given the importance of the vector longevity.

Chapter 6

6. General discussion

6.1 Summary of results

Our results concur with the importance of studying the ecological immunity of mosquitoes for a better understanding of malaria transmission and vector control. We found costs of immunity, physiological limits and complex interactions between benefits and costs. Besides, the interactions between larval environmental factors shape malaria transmission and host traits.

Evolution of immunity and malaria transmission

We found some physiological costs of immunity in chapter 2, which concurred with former studies [108] and helped explaining the variability of the immunity and the resistance to parasites. We did not found a clear genetic variation of inducing immune costs, but there were some negative correlations between the costs. For genotypes that had an efficient immune response against one type of antigen, it was costly to mount efficiently other immune responses. Besides, we found also a negative correlation between the cost and the efficacy of the melanisation response. It was similar to the smaller reduction in lifespan and reproductive success observed in aphid where the most protective endosymbiotic bacteria against parasitoids wasp represented the less costs [118].

These correlations influence the evolution of immunity in a more complex way, than a simple balance between costs and benefits for each immune response [114]. We would not have all immune responses at an intermediate efficacy, but instead a runaway selection for one immune response that would make others more costly and more difficult to maintain. However, we only looked at longevity and these correlations may depend on the immune pathways and the life history traits, as the efficacies of both immune response can be positively [115] or negatively correlated [116]. Besides, the efficacy of an immune response could

be positively correlated with the cost on fecundity [172] and negatively with the cost on longevity.

Furthermore, the cost of the melanisation response may less be due to the production of effectors than to the recognition of antigens and the stimulation of the melanisation response, as the cost on fecundity was lower for the highly melanised neutral Sephadex beads compared to the less melanised negatively charged Sephadex beads [107]. The recognition is an essential step and an immune response, like the melanisation, can be overloaded by an increasing immune challenge to detect. In chapter 3, mosquitoes when inoculated with several beads, prioritized the melanisation of one bead to a high degree, instead of melanising equally each bead. Indeed, the least melanised bead, in each mosquito, was melanised less than half as much as the best melanised one. There were some physiological limits to the immune efficacy, like the need to replenish phenoloxidase reserves observed in honeybees [154], and they may influence the evolution of resistance to malaria parasites.

There is more to immune responses' evolution than a simple balance between costs and benefits. There is a complex network of interactions and physiological limits that will determine the general immune efficacy and the resistance to parasites with large consequences for malaria transmission.

Larval environment and vector competence

There is an interaction between temperature and food during the larval development with an affect on adult vector competence. In chapter 4, vector competence was about a third lower when mosquitoes had only half of the standard diet as larvae. This effect of food varied with the temperature, as a low temperature was in favor or malaria transmission only at standard food. These findings complement the knowledge gained in an earlier study [161], where low diets had an impact on the oocyst load, and they highlight the caution needed, as environmental factors can be influenced by each other.

This influence of environmental factors was in part due to mosquito size, as mosquitoes were smaller and less competent [163, 185], when undernourished or reared at high temperatures [184]. However, after controlling for size, there was still an influence of food and temperature on vector competence.

Undernourishment leads to lower parasite survival and lower mosquito survival, probably because of the lower levels of resources stored [162]. Undernourishment could also lead to reduced resistance to parasites in mosquitoes [121, 124, 189], but the main explanation seem to be the lack of resources for the parasite's development [17, 161, 162]. Temperature on the other hand, had no effect on the mosquito survival but an effect on the parasite survival, at low temperature and low food.

The interaction of environmental factors influences the larval development with consequences on the vector competence but also on the correlations between life history traits. In chapter 5, we found a weak relationship between size and longevity, especially at 25°C. However, the relationship was negative, when the environmental factors had opposed effects on size [158, 160, 164, 169], and positive, when they had synergic effects. This relation between size and fitness may vary regarding environmental factors and also life history traits– eg. reproductive success, immunity or the resistance to parasites. Smaller *Aedes aegypti* mosquitoes may be more [185] or less [191] resistant to the dengue virus when obtained by varying density or the competition.

The influence of larval ecology on vector competence and the relations between life history traits is key to improve the climate-based modelling of malaria transmission.

6.2 Further perspectives of research

An avenue of research could be to use more natural variations of environmental factors, with more temporal variations of temperature for example, based on the increasing knowledge coming from the field [142, 192]. Besides, using more natural associations of vector and parasites could also improve the predictions and this could be done in the laboratory of in the field. However, this come with constraints and laboratory settings are still of great interest to test hypothesis and develop the conceptual framework of host parasite interactions in the case of vector borne diseases.

Following earlier studies [99, 101, 102] and some pilot studies, we would do further work on immunosuppression, as it is a puzzling component of host parasite interactions and immune responses variability.

Besides, we conducted some other pilot studies on the correlation between immunity and insecticide resistance and the associated consequences on the resistance to malaria parasites. This has been the subject of recent papers [193–195] and constitutes a challenging avenue of research. Indeed, there is growing insecticide resistance in the wild, with limited alternatives to bed nets and pyrethroids [35, 40, 196], while insecticide resistance could lead to increased susceptibility of mosquitoes to malaria parasites [193, 194].

6.3 Conclusion

The ecological immunity of mosquitoes may lead to a more complex vision of the evolution of immunity. The classic predictions of evolutionary ecology with a trade off between costs and benefits for each immune response may be too simple. Besides, environmental factors will influence the resistance to malaria parasites and the transmission of malaria, through their effects on the different stages of the mosquito life cycle. There is a need to improve further our understanding of host-parasite interactions for efficient vector control programs.

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