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Adaptation of mosquitoes to climate change and its impact on the transmission of a vector-borne pathogen

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Summary

With rising global mean temperatures, mosquito-borne disease such as malaria, dengue or Zika are prone to experience shifts in prevalence, intensity, and geographical range. This is largely due to the temperature dependence of both the mosquitoes and the parasites. Since mosquitoes are ectotherm, most of their development and physiology are strongly affected by temperature. Because of the biokinetics of enzymes, the thermal performance of insects is typically non-linear and left-skewed around an optimum. Consequently, very small shifts towards warmer temperatures can have dramatic effects. So far, most studies that described the relationship between mosquitoes, mosquito-borne transmission and temperature have been looking at phenotypic differences in life-history traits and transmission based on short-term experiment. But climate change is a long-term process that gives mosquitoes time to evolve to new environmental conditions.

In this thesis, we've sought to investigate the potential of adaptability of the yellow fever mosquito, *Aedes aegypti,* to changing temperatures and its underpinnings. In the first two chapters of this thesis, we evaluate and discuss how thermal acclimation influences different branches of the mosquito's immune system. In the following three chapters, we use an evolutionary approach to investigate the impact of thermal adaptation on several aspects of mosquito's vectorial capacity. By rearing mosquitoes over several generations at four different temperatures – 24°C, 26°C, 28°C and 30°C –, we've created 12 temperature-adapted lines. In chapter 3, we assess the impact of evolutionary thermal adaptation by measuring the thermal response of our lines regarding their larval development, adult body size and longevity. In chapter 4, we evaluate their anti-bacterial response and competence for dengue at different temperatures. And in chapter 5, we measure the thermal preference of cold and warm-adapted lines. The results of these experiments and their implication for the epidemiology of vector-borne diseases in a changing climate are discussed throughout the thesis. Overall, this work provides a broader understanding of the impact of climate warming on the thermal biology of mosquitoes and highlights the need to consider evolutionary changes to make reliable predictions.

Key words: Temperature, *Aedes aegypti*, thermal acclimation, thermal adaptation, immunity, vector competence, vectorial capacity

Résumé

Avec l'augmentation des températures moyennes mondiales, les maladies à transmission vectorielle telles que le paludisme, la dengue ou le Zika sont susceptibles de connaître des changements de prévalence, d'intensité et de répartition géographique. Cela est largement dû à la dépendance à la température à la fois des moustiques et des parasites. Étant donné que les moustiques sont des ectothermes, leur développement et leur physiologie sont fortement influencés par la température. De plus, en raison de la biocinétique des enzymes, la performance thermique des insectes prend une forme non linéaire et décalée vers la gauche autour d'un optimum. Par conséquent, de très petites variations vers des températures plus chaudes peuvent avoir des effets dramatiques. Jusqu'à présent, la plupart des études décrivant la relation entre les moustiques, la transmission de maladies vectorielles et la température ont examiné les différences phénotypiques dans les traits de l'histoire de vie et la transmission basée sur des expériences à court terme. Cependant, le changement climatique est un processus à long terme qui laisse aux moustiques le temps de s'adapter aux nouvelles conditions environnementales.

Dans cette thèse, nous avons cherché à étudier le potentiel d'adaptabilité du moustique tigre africain, *Aedes aegypti*, aux températures changeantes. Dans les deux premiers chapitres de cette thèse, nous évaluons et discutons de l'influence de l'acclimatation thermique sur différentes branches du système immunitaire du moustique. Dans les trois chapitres suivants, nous utilisons une approche évolutive pour étudier l'impact de l'adaptation thermique sur plusieurs aspects de la capacité vectorielle du moustique. En élevant des moustiques pendant plusieurs générations à quatre températures différentes - 24°C, 26°C, 28°C et 30°C -, nous avons créé 12 lignées adaptées à ces températures. Dans le chapitre 3, nous évaluons l'impact de l'adaptation thermique en mesurant la réponse thermique de nos lignées en ce qui concerne leur développement larvaire, leur taille corporelle adulte et leur longévité. Dans le chapitre 4, nous évaluons leur réponse antibactérienne et leur compétence pour la dengue à différentes températures. Et dans le chapitre 5, nous mesurons la préférence thermique des lignées adaptées au froid et au chaud. Les résultats de ces expériences et leurs implications pour l'épidémiologie des maladies vectorielles dans un climat changeant sont discutés tout au long de la thèse. Dans l'ensemble, ce travail offre une compréhension plus large de l'impact du réchauffement climatique sur la biologie thermique des moustiques et souligne la nécessité de prendre en compte les changements évolutifs pour effectuer des prédictions fiables.

Mots-clefs : Température, *Aedes aegypti*, acclimatation thermique, adaptation thermique, immunité, compétence vectorielle, capacité vectorielle

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General Introduction

Background / Global Burden of Vector-borne disease

Vector-borne diseases (VBDs) are a major cause of the worldwide burden of infectious diseases accounting for 17% of all cases and contributing to more than 700, 000 deaths annually (1,2). These diseases are caused by a wide range of pathogens including parasites, bacteria, and viruses. They are transmitted by vectors - most often hematophagous arthropods such as mosquitoes or ticks. Among mosquito-borne disease malaria and arbovirosis such as dengue, chikungunya and Zika are most prominent (3,4). With a staggering estimated 247 million cases worldwide, malaria claims the lives of more than 600,000 individuals each year, with a particularly devastating impact on children under the age of five (5). Among mosquito-borne viral diseases, dengue is the most rapidly spreading disease (6–9). In 2019 an estimate of 5.2 million cases and 36 000 deaths were reported (10). This number is probably a vast underestimation as many cases are asymptomatic and/or not formally reported to the WHO. The true number of worldwide dengue cases is likely to be 10 to 20 times higher (10). As of July 2023, already 3 million cases and 1500 dengue-related death have been signaled (11). The highest prevalence of VBD is experienced in tropical and sub-tropical countries with lower to middle incomes levels (2,10,12). As such, VDBs are considered to be a major socio-economic burden contributing to poverty and impact the economic development of at-risk regions (13).

In recent decades, there's been an increase in prevalence and shift in temporal and geographical distribution of VDBs (reviewed in (14)). For example, malaria infection has been shifting toward higher altitudes, its seasonality decreased, and it has been reemerging in temperature regions (15,16). Arboviral outbreaks have become more frequent as evidenced by the Zika epidemic in South America in 2015-2016, the chikungunya outbreak in Italy in 2017 or the current dengue epidemic in South America (17,18) and the geographical range of dengue has dramatically expanded over the last decades (9,19,20). Currently, more than 100 countries are endemic to dengue fever and cases in temperate regions such has North America and Europe are increasing in frequency (10,21). Recent projections estimated that 2.25 billion more people could be at risk of dengue by 2080 as compared to 2015, which would represent about 60% of the global population (22). Climate change is often named as a major driver of those changes in the epidemiology of VBDs. Although the relative importance of climate change compared to other non-climate drivers such as globalization, urbanization or control measure remains difficult to quantify and is under debate (12,23–25).

Anthropogenic-driven greenhouse gas emissions, largely stemming from the combustion of fossil fuel have cause the global mean temperature to increase by 1.1°C above preindustrial levels with most of the changes happening over the past 50 years (26). This rise in temperature has profound effects on our environment. Its impact is heterogeneous in space and time and has led to many region-specific

changes (e.g., (27). For instance, while the number of cold days and nights has dwindled, the number of warm days and nights has increased, so has the number of extreme climatic events such as heat waves, droughts, or floods (26). Finally, snow cover has dramatically decreased, leading to an accelerated rise of sea levels with colossal consequence for coastal environment (28). If the current trend in greenhouse emission continues an increase of mean global temperature by 4°C to 5°C above preindustrial levels can be expected (26,29). And although, within the frame of the Paris agreement many nations have pledged to maintain global average temperature below 2°C and ideally around 1.5°C above pre-industrial levels (30), a global warming of the temperatures by 2.5°C to 2.9°C or more is still expected by the end of this century (31).

Mosquito-borne disease transmission is the result of the complex interaction between hosts, vectors, pathogens, and their shared and respective environments. Warming and other manifestations of climate change might have profound implications on the dynamic and geographic spread of such diseases (reviewed in e.g., (25)). Variation in climatic factors will modulate the spatiotemporal distribution, performance and abundance of hosts, vectors, and pathogens (32). For instance, altered rainfall pattern are likely to provide more or less breeding sites for the vectors, hence altering vector abundance (33). A recent example is the current dengue epidemic in Peru where part of the increased burden is attributed to warmer and rainier weather linked to the tropical cyclone Yaku and the El Nino effect that affected the country during the spring (11,18). These extreme climatic events created optimal conditions for the breeding of the main dengue vector, *Aedes aegypti*, which resulted in enhanced transmission.

Changes in temperature are expected to influence most physiological and behavioral traits of the vector as well as the temperature-dependent developmental rate of the pathogens (Reviewed in e.g., (34). This is because both arthropods, including mosquitoes, and other vectors are ectotherms.

Thermal biology of mosquitoes

As ectotherms, mosquitoes are unable to control their body temperature and are directly affected by environmental temperature. Across ectotherm taxis, most physiological and life-histories traits display a non-linear, unimodal, and asymmetrical relationship to temperature (**Figure 1**) (35–37)**.** The performance of a given trait starts from zero, increases until reaching a maximum value or optimum, and then decreases back to zero. The two ends of the curve represent the critical thermal limits—minimum and maximum—while the difference between them represents the thermal tolerance range (38).

This unimodal relationship is predicted by metabolic processes and enzyme kinetics (35,36,39,40). According to metabolic theory of ecology, the rates of increase and decrease of the curve are

constrained (41). The rate of increase will be slower within the linear section, known as the Arrhenius section, while the rate of decrease above the optimum will be accelerated, resulting in an asymmetric curve (41). Consequently, very small increment beyond the thermal optimum will result in disproportionate effect on the thermal performance (38,41,42).

For traits such as development, reproduction and survival that have influence organismal fitness, the cost of going beyond the temperature of the thermal optimum is exceeding the cost going below (32,39,43–45). Hence, many speciess have evolved thermal performance curve with optima that are typically well above average environmental temperatures (44,46). In temperate regions, where temperature fluctuations are typically large and frequent, speciess tend to display large thermal safety margins (46–48). On the other hand, speciess that live in tropical environment with lesser temperature variation evolved a narrower thermal safety margin (43,48–50).

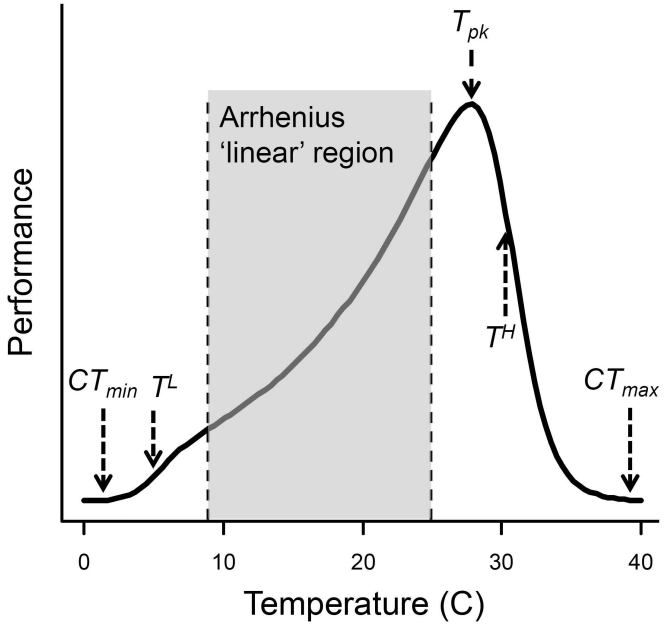

Figure 1: Theoretical illustration of a thermal performance curve. Source: Molnár et al 2017 (38)

Vectorial capacity and temperature

Vectorial capacity is typically used to describe the capacity of a vector to transmit an infectious agent at a given time, in a given environment (51). Vectorial capacity quantifies the average daily secondary cases arising from one primary case in a susceptible population (52). It includes five parameters relative to the vector and one to the pathogen. All these parameters are to some degree temperature dependent.

The classical Ross-McDonald equation (53) for vectorial capacity is as follow:

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

Where VC is vectorial capacity, m: the mean number of female mosquitoes per host, a: the average biting rate, b: the probability of transmission from mosquito to host, p: the mosquito's daily survival rate and n: the extrinsic incubation period (EIP) of the pathogen. This equation is useful in quantifying and predicting how mosquito traits contribute to the transmission of a pathogen. Ultimately, the dynamic and interplay among those traits in combination with traits relative to the host i.e., recovery rate and host susceptibility, will determine the transmission rate of a disease. For instance, as mosquitoes need to survive long enough to be infected and become infectious, the EIP of the parasite is heavily constrained by the vector's longevity (54). In the case of malaria, where the parasite's EIP is long, small reduction in longevity (8%) leads to significant reduction in vectorial capacity (80%) (55) . Similarly, the number of females per host is directly dependent on both the biting rate- mosquitoes need a blood meal to reproduce- and longevity- the longer females live, the more often they can lay eggs-. Within vectorial capacity there is also vector competence. Vector competence quantifies the ability of a vector to transmit a pathogen after exposure and will determine its probability of carrying an infectious bite (b) (51). For a mosquito to be competent it needs to be permissive to the entry, replication, dissemination, and release of the pathogen in the salivary glands (56). An important aspect of this is that the degree to which a vector is permissive or refractive for a parasite is highly dependent on its immune responses and microbiota (57,58).

While we will here focus on temperature it is noteworthy to mention that vectorial capacity is also dependent on other environmental conditions that affect the environment in which mosquitoes grow and live (59,60). Those include food availability (e.g., (61–69)), presence of predator or other pathogens (e.g., (70–74) and exposure to xenobiotics such as insecticides (e.g., (75–81).

For mosquitoes, virtually all life-history traits including traits influencing vectorial capacity are temperature-dependent (32). Although the effects of temperature on life-history trait are dependent on the combination of mean temperatures and magnitude of fluctuating temperature (42,82–84) – i.e., daily temperature fluctuation -we here only focus on the effect of constant temperature on the yellow fever mosquito *Aedes aegypti.*

Mosquito larvae and pupae are strictly aquatic and submitted to temperature variation in their breeding sites from which they have little opportunity to escape. Typically, larval developmental time decreases with temperature until it reaches the upper thermal limit for survival (85–88). For *Aedes aegypti*, the optimal temperature for larvae survival in laboratory is 26°C but they can survive at temperatures between 16°C and 34°C or 38°C (82,85). The optimal temperature for developmental time is much higher at 32°C. Variation in temperature during larval stage will carry-over to adult with effects on several traits such as fecundity and lifespan (59,89). This is largely due to the correlation between these traits and body size (90). Larvae exposed to colder temperature develop slower, accumulate more resources, and generally morph into bigger adults and vice versa at warmer temperatures (87,91–96). The effect of body size on other traits such as lifespan, biting rate and immunocompetence are open to discussion (61,91,97,98). For example, while some studies report that smaller individuals tend to live less long, and blood-feed more often other conflicting results were obtained elsewhere (99–101). Temperature also directly impacts mosquito lifespan and fecundity. Temperature extreme of 16°C and 36°C have been reported as critical temperature for both traits in *Aedes aegypti.* Optimal temperature for longevity has been estimated between 21°C and 25°C for a lifespan of 38 days (32,102). Optimal temperature for fecundity - here defined as the maximum number of eggs laid - has been estimated between 26 °C and 30°C (32,82). Additionally, the length of the gonotrophic cycle – that is the time between each blood meal and egg laying event- decreases with increasing temperature (82). This will influence the number of generations produced during one lifespan and the number of contacts between mosquitos and hosts. Finally, temperature also interacts with vector competence for it influence both the parasites EIP and the mosquito's ability to fight off an infection i.e., its immune functions. Temperature related changes in immune functions are particularly complex as they do not scale with temperature (103). For example, previous work on *Anopheles stephensi* has evidenced that temperatures that increase the expression of the anti-microbial peptide (AMP) *defensin* were less favorable for the expression of the AMP *cecropin* and vice-versa (104). Similar observations were made for the temperature-dependent expression of nitric oxidase synthase and phenol oxidase-based melanization (104). Differential transcriptomic and immune gene expression profiles have been described for mosquitoes infected with Zika virus and chikungunya virus at different temperature (105,106). While most evidence point toward an increased

transmission potential of arboviruses as temperature increases, the opposite has been observed for some few specific mosquito-virus interaction. Enhanced vector competence of *Aedes aegypti* for the chikungunya virus has been associated with lower temperatures (106). In summary, since temperature impacts mosquito biology, physiology, and behaviors, it is a key abiotic determinant in the dynamics of mosquito-borne transmission (107).

Thermal adaptation

Mosquitoes have evolved several strategies that allow them to thrive in spatially and temporally variable thermal environment and to avoid thermal stress. For instance, the synthesis of heat shock protein enables mosquitoes to cope with periodic short-term temperature increase (108). For mosquitoes found in temperate region such as *Culex pipiens s.l., Culex tarsalis* or *Culex quiquefasciatus* the ability to diapause has been suggested as an adaptation to survive cold seasons (109). Similarly, diapause along with shifts in thermal tolerance are often considered as key factors that facilitated the rapid establishment of the highly invasive Asian tiger mosquito, *Aedes albopictus* in temperate regions (110). Other mechanisms to cope with thermal heterogeneity of the environment include thermoregulation, thermal preference, or adjustment of behavioral activity (108,111–114). As global temperature increases in face of rapid anthropogenic climate warming the distribution and performance as vector of mosquito populations might change for temperatures might exceed their critical thermal limits. Population might persist through various mechanisms, one of which is evolutionary thermal adaptation (115,116).

Evolutionary thermal adaptation is likely for mosquitoes as they are short-lived organisms with high population growth rates (39,40,117,118). Speciess like *Aedes aegypti*, that predominantly inhabit tropical environments with minimal seasonal temperature fluctuations, especially hold potential for evolutionary thermal adaptation (114). This is due to their narrower thermal tolerance range compared to speciess located in more diverse and variable temperate environments (118). The potential for evolutionary thermal adaptation, the form it may take and its effect on performance in mosquitoes remains poorly understood. Potential thermal adaptation could result in shifts in thermal optimum for one or more traits, in broadening of the thermal limits or in increases in heat or cold tolerance (32,116,119,120).

Thesis aims

Throughout this thesis, I was interested in the response to temperature of the yellow fever mosquito, *Aedes aegypti*. I tried to understand how long-term changes in temperature influence the phenotypic response of mosquito traits involved in vectorial capacity. In the first two chapter of this thesis, I

investigated the influence of shared thermal environments of larvae and adults on their immune response - **Chapter 1** - and explored the interplay among these responses - **Chapter 2**.

Temperature modulates the immunocompetence of mosquitoes. However, its effects are complex and vary among different branches of the immune system (121). Furthermore, although the immune response is measured in adults, it is also influenced by the larval environment through carry-over effects (59,80,89,122). It is thus important to consider changes in environmental temperature in both larval and adult stages. In the **first chapter** of this thesis, I tested the immune response of mosquitoes reared and maintained at four different temperatures – 24°C, 26°C, 28°C and 30°C. Using micro-injection of negatively charged Sephadex beads, *E. coli* bacteria, and medium, I stimulated and measured their melanization and anti-bacterial responses, as well as their survival post-injury. In the **second chapter** of this thesis, I explored the interplay between the melanization and anti-bacterial response at different temperatures. Immunocompetence is a complex trait that involves several mechanisms that interact with one another. The interplay between different branches of the immune system is rarely considered when assessing immunocompetence. Due to physiological constraint, the activation of one immune response could be impeded to the favor of another response. I've tested this idea and how temperature might modulate the interplay with two experiments. I stimulated mosquitoes that were reared and maintained at four temperatures – 24°C, 26°C, 28°C and 30°C- with either a negatively charged Sephadex bead, *E. coli* bacteria or with a combination of the two -that is a negatively charged Sephadex bead and *E.coli* bacteria. I evaluated whether the type of immune challenge (presence or absence of a simultaneous challenge), experimental temperature and their interaction influenced the melanization response (experiment 1) or the anti-bacterial response (experiment 2).

With the **next three chapters**, I've tried to understand the long-term response of mosquitoes to temperature. As temperature increases with global warming, the distribution and performance of vector populations and the transmission dynamics of vector-borne pathogens are expected to change (32,123,124). So far, most of the information concerning the effects of temperature on vectors and vector-borne transmission comes from short-term experiments. But climate change is a long-term process that gives mosquitoes time to evolve and adapt to new environmental conditions. Yet, the adaptation potential to temperature of mosquito remains poorly understood. In particular, it is currently unknown whether once adapted mosquitoes will display the same phenotypic patterns as in short-term experiments and whether the phenotypic impact of evolutionary changes is important for transmission.

To evaluate the evolutionary potential for thermal adaptation in *Aedes aegypti* and its consequences I created 12 evolutionary lines at four different temperatures – 24°C, 26°C, 28°C and 30°C- and

maintained them for more than 16 generations. In **Chapter 3,** I evaluated the thermal response of adapted lines at seven different ambient temperatures ranging between 24°C and 34°C. After rearing mosquitoes from each adaptation line at each of the seven experimental temperatures, I recorded several of their life-history traits – Larval survival and developmental time, sex ratio, body size and longevity- and evaluated the effects of adaptation temperature, experimental temperature, and their interaction. In **Chapter 4,** I measured the immune response and vector competence of temperature-adapted lines. In a first experiment, I measured the ability of adapted lines to limit bacterial growth when reared and maintained at different temperatures. To do so, I reared two lines adapted to each of the four adaptation temperatures at four different temperatures – 24°C, 26°C, 28°C, and 30°C. I challenged them with *E. coli* bacteria using micro-injection and measured the bacterial load 48 hours after the injection. In a second experiment, I tested the vector competence for dengue the lines adapted to 24°C, 28°C and 30°C. To do so, I provided adapted mosquitoes, which were reared at three different temperatures – 24°C, 28°C, and 30°C, with the opportunity to take a dengue-infectious blood meal. To evaluate their vector competence, I recorded the infection prevalence, dissemination prevalence and disseminated viral load. In **Chapter 5,** I tested whether cold and warm-adapted mosquitoes displayed different thermal preferences. Thermal preference enables mosquitoes to select thermal microhabitat that help them cope with heterogeneous thermal environment and/or warming temperatures (113,114). For this experiment, I only considered lines that were adapted to 24°C and 30°C. I tested the thermal preference of adapted mosquitoes that were reared at either 24°C or 30°C in a thermal gradient ranging between 19°C and 35°C. Mosquitoes were video tracked for 15 minutes. I assessed the time spent at each temperature as well as flying activity in function of adaptation and rearing temperature and its interaction.

Experimental system

The yellow fever mosquito: *Aedes aegypti*

Aedes aegypti is an important vector of mosquito-borne disease, in particular arboviruses. It is the main vector of yellow fever and dengue and is involved in the transmission of other diseases such as chikungunya, Zika or chicken malaria (10,11). It originated in Africa and is now ubiquitous in tropical and subtropical regions around the world (125). Its domestic polytype exhibits a preference for urban settings, thriving in close proximity to human populations, where it finds ample breeding sites and hosts for its blood meals (126).

As a holometabolous insect, its life cycle is divided into four stages: eggs, larvae, pupae, and adults **(Figure 2)**. The first three stages are aquatic, and the adult stage is non-aquatic. Females lay eggs into natural or artificial water container where larvae will develop feeding on microorganism and other

organic compound in the water (127). Larvae will molt three times before transforming into pupae. Larval development usually lasts seven days but can range from six to 31 days depending on environmental conditions (128–130). Pupae are mobile, non-feeding and take about two days to develop before metamorphosing into adults. Within two- three days after emergence male and female will mate. Female usually mate only once since a single insemination contains sufficient sperm for the female to lay eggs throughout her lifespan (131). Being an anautogenous organism, female mosquito needs a blood meal to lay eggs. The frequency of oviposition is determined by the length of the gonotrophic cycle which includes the search of a host, ingestion of a blood-meal, digestion of the blood, maturation of the ovaries and oviposition (132). Aside from its importance for public health, *Aedes aegypti* is a particularly well-suited species for laboratory work for their eggs can be stored for a long period and their hatching synchronized using a partial vacuum (133). This allows for experiment on individuals of the same age regardless of variation in developmental time due to environmental conditions – here temperature. For this thesis, I worked with the UGAL/Rockefeller strain of *Aedes aegypti* (134) provided by Patrick Guérin. This strain was established in 1970 and has been maintained at the University of Neuchâtel for many years under standard insectary conditions (26.5 ± 0.5 °C and 70 ± 5% humidity, with a 12:12 photoperiod).

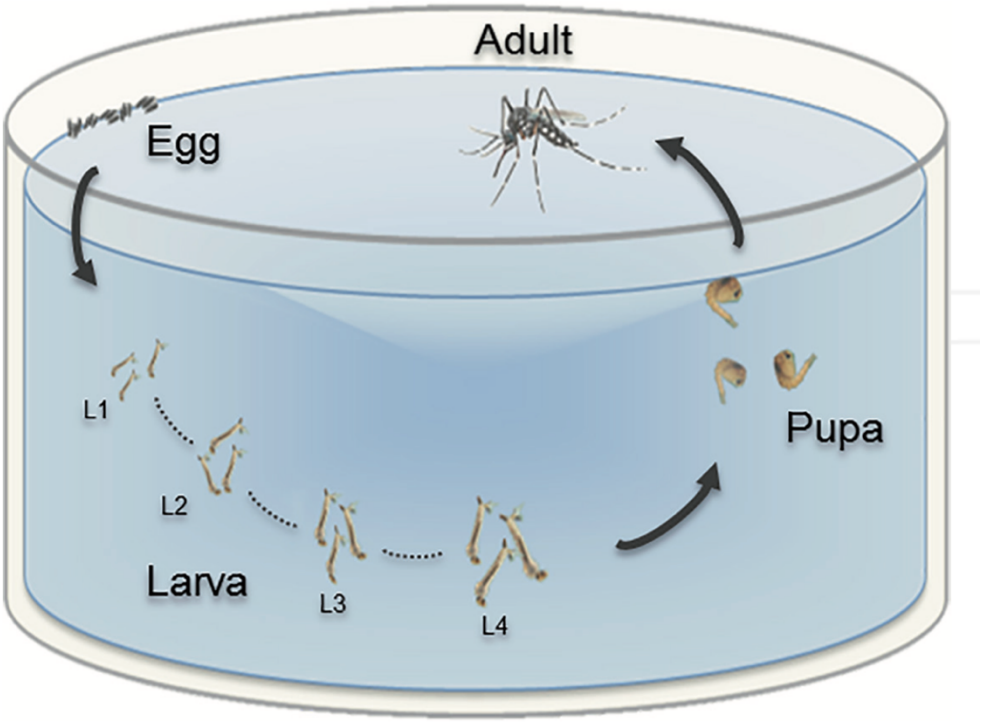

Figure 2: Generalized life cycle of *Aedes* mosquitoes. *Aedes* mosquitoes are holometabolous organisms und undergo four stages during their life cycle: Egg, larva, pupa, and adult. Eggs are primarily laid in water-filled artificial containers such as plant vases or tires. The development from larvae to pupae occurs in aquatic environments, after full metamorphosis, the adults are aerial. Source: Chouin-Carneiro et al 2017 (135)

Thesis overview

The main goal of this thesis was to investigate the short-term and long-term effects of temperature on the immunity and life history of the yellow fever mosquito, *Aedes aegypti*. The results of this thesis hopefully provide insight into how mosquitoes might cope with changing temperatures in the face of rapid anthropogenically driven climate warming.

This thesis is composed of five main projects reported in the following five chapters:

- **Chapter 1:** "Impact of shared thermal environment between mosquito larvae and adults on their immune response "
- **Chapter 2:** "Interaction between melanization and anti-bacterial responses of the mosquito *Aedes aegypti* at four temperatures "
- **Chapter 3:** "Thermal adaptation in a major vector species: Adaptation to temperature impacts the thermal response of the Yellow fever mosquito"
- **Chapter 4:** "Temperature sensitive immune function and vector competence for dengue is unaffected by thermal adaptation in the Yellow fever mosquito"
- **Chapter 5:** "Unexpected behavioural adaptation of yellow fever mosquitoes in response to high temperatures "

Chapter 1

Impact of shared thermal environment between mosquito larvae and adults on their immune response

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Abstract

Given that mosquitoes are ectotherms, temperature plays a pivotal role in shaping their physiological responses including their immunity. Its impact on immune functions has typically only been studied in adults maintained under different thermal conditions. But since carry over effects between larval and adult stage are common, the temperature at which larvae are reared is likely to have long-lasting effects on the adults. In this present study, we investigated the influence of temperature on the immune function of the yellow fever mosquito, *Aedes aegypti*. We assessed the thermal response of three aspects of immunity in mosquitoes that were reared and maintained at four different temperatures. We found that mosquitoes acclimated to warmer temperatures (1) melanized negatively charge Sephadex bead to a lower degree, (2) were less likely to clear an *E. coli* infection and (3) were more likely to die after an injury, than mosquitoes acclimated to lower temperatures. Together, our results indicate that the immunocompetence of mosquitoes might be impaired as environmental temperatures increase which is relevant for example in the context of climate change.

Introduction

The ability of mosquitoes to resist infections and transmit pathogens is determined in large part by their immune system (136,137). The strength of mosquitoes' immune response is strongly impacted by their genetic background and their environment (136,138–140). Indeed, several studies have emphasized that factors such as food conditions, exposition to insecticides, microbiota or temperatures affect the immunocompetence of mosquitoes (82,104,122,141–144). Temperature is considered as one of the most important abiotic factors influencing the biological processes, physiological functions, and performance of poikilothermic invertebrates, including mosquitoes. It is hence not surprising that immune functions of vector mosquitoes show temperature-dependent patterns. For example, recent studies have shown differential immune gene expressing profile at different temperatures in *Aedes aegypti* infected with the Zika virus and the chikungunya virus, (105,106). But although, most studies have only considered the effect of temperature on adult stages, the temperature experienced by larval stages might be equally important in shaping immune functions and vector competence. This is because, innate immunity, infection and escape barriers are formed during larval development (63,79,145–147). For instance, rearing *Aedes albopictus* larvae at cool temperatures reduces the dissemination of dengue-1 virus regardless of the temperature at which adults are being held (148).

We here set out to investigate how shared thermal environment between larvae and adults influences the immunocompetence of the yellow fever mosquito *Aedes aegypti*. To do so, we reared and maintained mosquitoes at four different temperatures - 24°C, 26°C, 28°C and 30°C - and assessed three aspects of their immunity: (1) melanization response, (2) anti-bacterial response, (3) survival post-injury.

The melanization response is an encapsulation response in mosquitoes that has been linked to the ability of mosquitoes to clear and limit the dissemination of many pathogens including plasmodium and arboviruses (121,149,150). It is a single component humoral response that involves the triggering of the phenol oxidase cascade that results in the synthesis of melanin that is deposited around a foreign body (151). The anti-bacterial response is a composite response that can involve several branches of the immune system (121). To supress or limit bacterial growth mosquitoes can rely on the production of anti-microbial peptides such as defensin, the use of reactive oxygen speciess, phagocytosis, and melanization (152). Finally, an injury is sufficient to activate an immune response by the recognition of molecular by-product of tissue damage (153). It has been linked to memory-like responses that offer increased protection to subsequent infection later in life and enhanced survival (154).

Materials and Methods

Experimental design

For this study, we used the UGAL strain (134) of *Aedes aegypti* which has been maintained in our laboratory at 26° ± 0.5 ° C, 70 % ± 10% and 12h:12h light:dark cycle since 2012.

We assessed the phenotypic effect of temperature on three aspects of the mosquitoes' immunity: 1. melanization response, 2. anti-bacterial response and 3. survival post-injury. The three measurements of immunity were considered as separate experiment whereas larvae and adults were reared and maintained at four different temperatures -24°C, 26°C, 28°C and 30°C- using the same protocol.

Mosquito rearing

Since developmental time in mosquitoes in temperature dependent, we staggered the hatching of eggs across four days to ensure that adult emerged at the same time regardless of their rearing temperature. Dried eggs were rehydrated in ddH_2O for one hour before being hatched under reduced-pression vacuum for 30 minutes. Six hours later, 1440 freshly hatched larvae were individually placed into 12-wells plates filled with three ml of deionized water. 360 larvae in 30-well plates were distributed among four climate-controlled incubation chambers set to 24°C, 26°C, 28°C and 30°C and to 70 % ± 10% relative humidity and a 12h:12h light:dark cycle.

Larvae were fed daily with a predetermined dosage of Tetramin Baby® fish food according to the standard of our laboratory. Each larva received 0.06, 0.08, 0.16, 0.32 and 0.6 mg at ages zero, one, two, three, four and five or more days, respectively. Pupae were transferred into 50ml Falcon™ tube filled with 10ml of deionized water and covered with mosquito netting. A cotton ball soaked into 6% sugar solution was placed and replaced daily on each falcon tube. After emergence, males and females were visually sorted. Males were discarded and 45 to 55 females were retained at each temperature for an additional four to five days in preparation for each immune challenge.

Immune assay

Melanization response

Melanization ability was assessed by inoculating five-days-old females with a negatively charged carboxymethyl Sephadex C-25 bead as described in (155). Briefly, on two consecutive days, we moved 25 females aged four or five days at each temperature into an empty 20ml Falcon™ tube. To immobilize the mosquitoes, we placed the empty Falcon™ tube on ice for ten minutes. We then injected a single bead into the thorax of the mosquito using a microcapillary. Beads range between 50 and 130um in diameter; we visually chose the smallest one for the injection. Injected females were transferred individually into a plastic cup filled to one-third with a 6% sugar solution, lined with filter

paper, embedded with a small petri dish, and covered with mesh. For the next 48 hours, injected mosquitoes were kept at the rearing temperature. After which, surviving individuals were frozen until further manipulation. Individuals that had died within that timeframe were counted and discarded.

Frozen mosquitoes were dissected in a 0.1% methyl green-colored solution and a picture of the recovered beads was taken under a light microscope with a 20x magnification. We quantitatively assessed the melanization response by estimating the amount of melanin deposited on each bead using the software ImageJ version 2.3.0 (156). For each picture, we filtered out the spectrum of unmelanized parts of the bead and measured the remaining grey value of the bead. We scaled the grey values between zero for unmelanized beads and one for fully melanized ones. Additionally, using ImageJ, we also measured the diameter of each bead as well as the length of wings from the tip of the wing to the distal end of the alula.

Anti-bacterial response

We assessed the anti-bacterial response by measuring the growth of ampicillin-resistant *E. coli* (dh5 alpha strain) within mosquitoes as described in Hauser et al 2020 (80). Every day, we prepared a bacterial solution by serial diluting it until it contained 17.5 $*10^6$ *E. coli*. To do so, we compared the absorbance at 600nm of a bacterial solution that had been grown overnight at 37°C in Luria-Bertani broth supplemented with 150ug/ml of ampicillin with a previously established standard curve based on known concentration of *E. coli*. To prevent further bacterial growth, the prepared solution was kept on ice immediately after being prepared and until being injected in mosquitoes.

On two consecutive days, we moved 25 four to five days old females into an empty 20ml Falcon ™ tube. We chilled those females on ice for ten minutes before inoculating them in the thorax with 0.2ul of bacterial solution equivalent to 3500 *E. coli* bacteria. Following the injection mosquitoes were transferred individually into a plastic cup and maintained at their rearing temperature as described above. 48 hours after the inoculation, we measured bacterial load in survivors. For this, we moved the mosquitoes into 1.5ml Eppendorf ™ tubes and crushed them using a micro-pestle in 200ul of Luria-Bertani medium supplemented with 150ug/ml of ampicillin. The homogenate was diluted 20-fold, and 100ul of the solution was spread on LB-agar plates supplemented with 150ug/ml of ampicillin and 250ug/ml of amphotericin B. The agar plates were incubated overnight at 37°C. Bacterial colonies were subsequently counted and the corresponding bacterial count was calculated. We used the number of bacteria as an inverse measure of the anti-bacterial response.

Survival post-injury

To assess survival post-injury, we injected female mosquitoes with a saline solution and monitored their survival for seven days post-injection. On two consecutive days, we moved 25 females aged four

to five days per temperature group into an empty 20ml Falcon ™ tube. Transferred females were chilled on ice for ten minutes before being injected with 0.2ul of sterile saline solution. The composition of the saline solution was as follow: 1.3 mM NaCl, 0.5 mM KCl, and 0.2 mM $CaCl_2$ in ddH_20 at pH: 6.8. 10ml of the saline solution was autoclaved on the night before the injection. Injured females were moved into a plastic cup as described above and maintained at their rearing temperature. After 48 hours, all surviving mosquitoes were transferred into a fresh cup similarly lined with filter paper, embedded with a petri dish, and closed with mosquito netting but filled to 1/3 with deionized water. On each following day, a fresh cotton wool soaked into a 6% sugar solution was placed on top of every cup. Mortality was assessed and recorded daily until all individuals had died.

Statistical analysis

All analyses were done using R for MacOS version 1.4.10 (157) . We analyzed each immune response separately. All analyses included temperature as a factor with four levels and day of injection as a factor with two levels. The significance of each factor was determined with the type II *Anova* function of the *car* package version 30-10 (158). Contrast analysis was done using the Estimated Marginal Means (*emmeans*) function of the *emmeans* package version 1.7.2 (159). P-values were adjusted using the *Fdr* method.

Melanization response

Differences in melanization were tested by comparing the grey values of the beads at each temperature using a linear model assuming a normal distribution of errors. In addition to temperature, we added the size of the bead, wing length (as a proxy for body size) and the day of injection as co-variables. Normality of the residuals was assessed visually with a *qqplot* and homoscedasticity was tested with a *Breusch-Pagan* test (*bptest* function of the *lmtest* library R version 0.9 3 8 (160).

Anti-bacterial response

We first analyzed the proportion of individuals that had cleared themselves from the bacterial infection as a function of temperature with a generalized linear model (GLM) with a binomial distribution. Within the mosquitoes that had not cleared the infection, we next analyzed bacterial load with a linear model. To achieve normality of the residuals we used the log10 values of bacterial load. Normality of the residuals was assessed visually with a *qqplot* and homoscedasticity was tested with a *Breusch-Pagan* test (*bptest* function of the *lmtest* library R version 0.9 3 8 (160). Both analyses included the day of injection as a co-variable.

Survival post-injury

Survival post-injury at different temperatures was analyzed using a Cox proportional hazard model from the *survival* package version 3.5-7 (161). Day zero was set at the day of the injection and all individuals that were alive seven days after the inoculation were censored. Temperature, as a factor with four levels (24°C, 26°C, 28°C and 30°C), was included as an explanatory variable and day of the injection, as a factor with two levels (day 1 or day 2), as a co-variable. The assumption of proportional hazard ratio was tested using the *cox.zph* function from the survival package.

Results

Melanization

We inoculated 50 females at 24°C, 28°C and 30°C. At 26°C, we inoculated 40 females. Of the 190 females total that we challenged with a negatively charged Sephadex bead, nine (4.9%) died within 48 hours of the injection. Mortality was independent of temperature. We retrieved 164 beads from the 181 surviving females that were processed.

All retrieved beads were at least partially melanized. The grey values of the beads varied among temperatures ($F = 5.69$, $df = 3$, $P < 0.001$) (**Figure 1**). Melanization values were higher at 24°C, (0.56 ± 0.049) (Mean ± 95% ci) than at 28°C, (0.42 ± 0.039) and 30°C, (0.47 ± 0.061) (**Table 1**). The grey value of the beads at 26°C fell in between (0.49 ± 0.041). Bead size ($F = 0.0001$, $df = 1$, $p = 0.998$), wing length (as a proxy for body size) ($F = 0.17$, $df = 1$, $p = 0.677$) and injection day ($F= 0.04$, $df =1$, $p = 0.8333$) did not significantly influence the grey values of the beads.

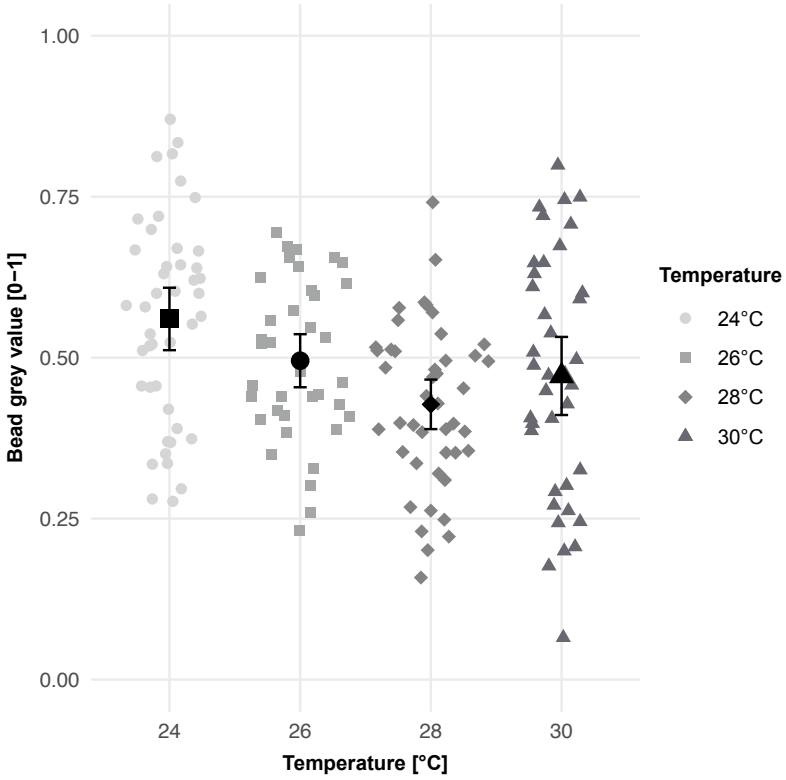

Figure 1: Illustrated here is the amount of melanin deposited on beads 48 hours after being injected into adult *Aedes aegypti* females at four temperatures (24°C, 26°C, 28°C and 30°C). The mosquitoes were inoculated four days after emergence and beads recovered 48 hours after the injection. The grey value was rescaled so that "0 "means that the bead is white, while a grey value of "1 "means that the bead is entirely black. The sample sizes are from left to right: $N_{24°C}$ = 44, $N_{26°C}$ = 37, $N_{28°C}$ = 44, $N_{30°C}$ = 39. Error bars show the 95% confidence interval around the mean.

Anti-bacterial response

We injected 3500 *E. coli* in 50 females at 24°C, 28°C and 30°C; at 26°C we inoculated 48 females. 14 mosquitoes (one at 24°C, three at 26°C, two at 28°C and eight at 30°C) died in the first 48 hours after the injection. Overall, 25% (95% ci: 19% - 32%) of the surviving females had cleared themselves from the infection. This proportion differed among temperature (χ^2 = 11.04, df = 3, p = 0.011) (**Figure 2a**). In particular, individuals reared and tested at lower temperature were twice as likely to clear the bacteria (24°C : 33% (95% ci : 12 %-16%) ; 26°C: 38% (95%ci: 14%-16%) than those tested at higher temperature (28°C : 15% (95% ci: 8%-14%); 30°C: 14% (95%ci: 8%-15%) (**Table 1**). Temperature did not affect bacterial load in the 138 mosquitoes that harbored *E. coli* 48 hours after the injection (F =1.99, df = 3, p = 0.117) (**Figure 2b**). Neither bacterial clearance (χ^2 = 0.008, df = 1, p = 0.929), nor bacterial load (F = 0.33, df =1, p = 0.565) were significantly impacted by the day of the injection.

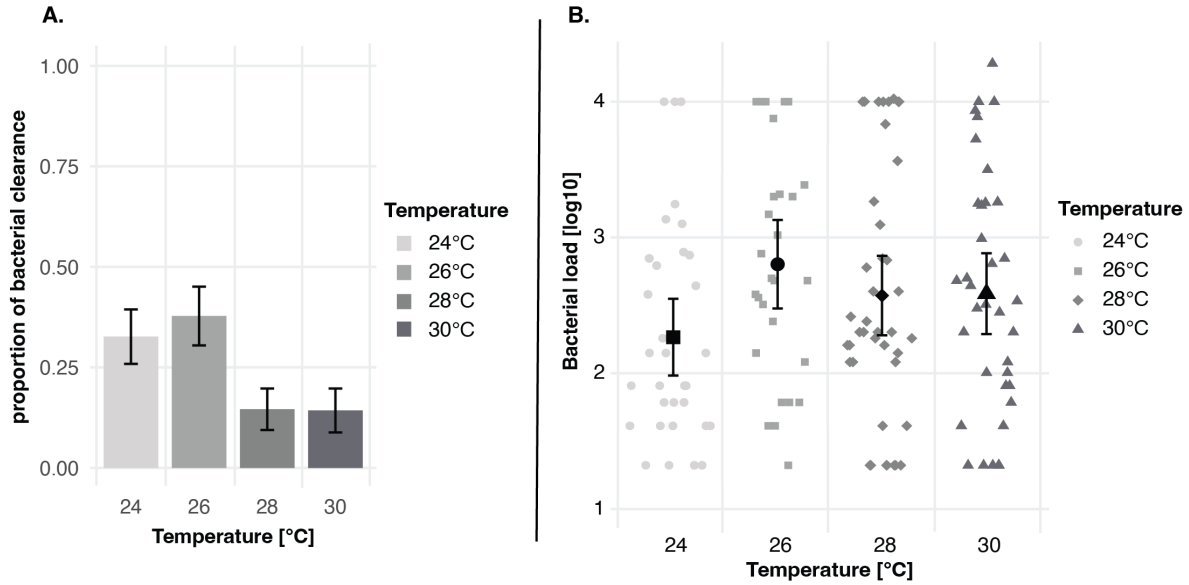

Figure 2: Illustrated here A. the proportions of individual that had cleared themselves from the bacterial infection 48 hours after the injection at four temperatures (24°, 26°, 28° and 30°C) and B. Bacterial load on a log10 scale in those individual that harbored *E. coli* 48 hours after the injection according to temperature (24°C, 26°C, 28°C and 30°C). Bacterial load was assessed by spreading of the mosquitoes on LB-agar plate and counting of the Colony Forming Units (CFUs) 48 hours after the injection. As a proxy for anti-bacterial response, the lower the bacterial load the higher the specific immune response. Samples sizes from left to right are: $N_{24°C} = 49$, $N_{26°C} = 45$, $N_{28°C} = 48$, $N_{30°C} = 42$. Error bars show the 95% confidence interval around the mean.

Survival post-injury

Across all temperatures, between 72% and 41% of all individuals were alive seven days after the injury. Temperature influenced the survival curves of the different groups ($\chi^2 = 13.8616$, df = 3, p = 0.0031) (**Figure 3**). About twice has many individuals reared and challenged at 30°C died within seven days of the injection (59% ± 15%) (Mean ± 95% ci) than at any other temperatures (24°C: 28% ± 12.9%; 26°C: 31 % ± 15%; and 28°C: 31% ± 13%) (**Table 1**). The day on which females were injected was not significant ($\chi^2 = 0.3091$, df = 1, p = 0.5782).

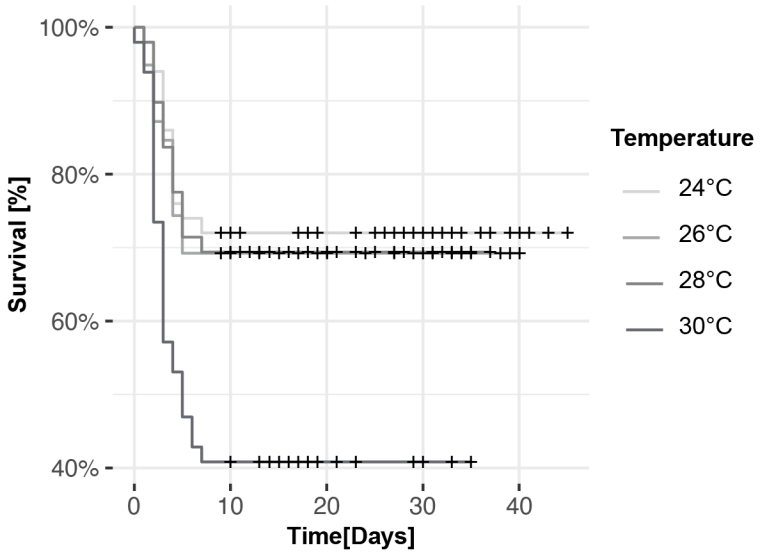

Figure 3: Illustrated here are the survival curves post-injury of female mosquitoes reared and maintained at four temperatures (24°C, 26°C, 28°C and 30°C). Day zero was set as the day of the injection that is four to five days after emergence. Surviving mosquitoes were censored seven days after the injury and here marked with a cross. The samples sizes are: $N_{24°C}$ = 50, $N_{26°C}$ = 39, $N_{28°C}$ = 49, $N_{30°C}$ = 49.

Table 1: Results of the post-hoc tests (Fdr) that compares 1. The grey values of the beads (measure of the melanization response) 2. Bacterial clearance (measure of the anti-bacterial response) 48 hours post challenge and 3. Survival seven days post-injury at four different temperatures. Significance is marked in bold and with an asterix.

		Melanization			**Bacterial Clearance**		**Survival**	
	Contrast	*t. ratio*	*df*	*p. value*	*z. ratio*	*p. value*	*z. ratio*	*p. value*
Temperature [°C]	24-26	1.887	157	0.2378	0.523	0.6013	-0.356	0.9247
	24-28	4.052	157	**0.0005 ***	-2.047	**0.0406 ***	-0.292	0.9247
	24-30	2.632	157	**0.0457 ***	-1.992	**0.0464 ***	-3.127	**0.0106 ***
	26-28	1.968	157	0.2047	-2.481	**0.0131 ***	-0.082	0.9350
	26-30	0.671	157	0.9079	-2.406	**0.0161 ***	-2.555	**0.0213 ***
	28-30	-1.304	157	0.562	-0.039	0.969	-2.859	**0.0128 ***

Discussion

Throughout our experiments, the immune response of *Aedes aegypti* was impaired at higher temperature. Indeed, the melanization, bacterial clearance and survival post-injury were higher in mosquitoes that were acclimated and tested at cooler temperatures than at warmer temperatures.

The degree of melanization of beads injected in mosquitoes reared and maintained at 24°C was 1.2 and 1.3 times higher than in mosquitoes reared and maintained at 28°C and 30°C. This result corroborates with similar studies on *Anopheles gambiae* (162) and *Anopheles stephensi* (104). Melanization, which is the production and deposition of melanin, serves multiple functions beyond

just immunity, such as making eggs more resilient (163–166). This could contribute to the observed stronger melanization response at cooler temperatures.

The proportion of mosquitoes that had entirely cleared themselves from the bacterial infection was more than twice as high in females that were reared and maintained at 24°C and 26°C than in those reared and maintained at 28°C and 30°C. In *Aedes aegypti*, phagocytosis is the most prominent pathway involved in the immune response against *E. coli* (152). Previous work on *Anopheles stephensi* have linked a higher phagocytosis index at cooler temperatures (104). Interestingly, we detected no effect of temperature on bacterial load in individuals who did not clear the infection after 48 hours. This might be related to the predominant action of anti-microbial peptides (AMPs) against persistent bacterial infection (167). In previous work, the expression of several AMP such as cecropin was temperature independent (103,104).

Finally, survival post-injury was drastically lower at our highest temperature. However, temperature also directly influences survival with intermediate temperatures linked to increased longevity (102) . It is thus unclear whether the observed results reflect an impaired capacity of mosquitoes to recover from an injury or the effect of temperature on longevity.

Together our results suggest that increasing temperature impair the phenotypic immunocompetence of *Aedes aegypti*. As metabolic rate and enzymatic activity tends to increase with temperature within the thermal tolerance range of mosquitoes, this result seems counter-intuitive (168). Recent studies however have reported that the expression of many genes, including immune related genes are altered by ambient temperature in a way that might impair immune functions at higher temperatures (105,106). An alternative explanative may be related to the carry over effects between larvae and adults and energetic cost associated to immunity. Mosquitoes reared at higher temperatures tend to grow faster (89,169–171). Accelerated development of larvae at warmer temperatures can interfere with nutriment intake and accumulation (95,172). This leads to smaller adults with smaller energetic reserves which is likely to influence their biological processes, including diminishing their immune functions as innate immunity and infection barriers are developed during larval stages (63,145). This was observed when larvae were undernourished either due to a low diet environment or when reared in competition (80,145,146,173,174).

In summary, rearing mosquitoes at warmer temperature may lead to smaller females that are less immunocompetent than their larger counterparts reared at cooler temperatures adults (175).

Chapter 2

Interaction between melanization and anti-bacterial responses of the mosquito Aedes aegypti at four temperatures

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Abstract

One aspect of immunocompetence that is often neglected is that different immune responses are not deployed individually but as a concerted effort of the immune system. Assuming that resource allocation for immunity is limited, the immune system may physiologically constrain itself by partially or fully suppressing one response in favor of another. We tested this idea in the yellow fever mosquito, *Aedes aegypti*, by stimulating its melanization and anti-bacterial responses in the presence and absence of the other response. Additionally, since the immune responses of insects is strongly influenced by environmental factors, we also assessed how temperature modulates the interplay between the two responses. We found while the anti-bacterial response was impeded by a simultaneous melanization challenge, the melanization response was enhanced by the presence of bacteria. In both cases, temperature modulated the interaction between the two branches of the immune system. Gaining better understanding of the dynamics of the immune system and its relationship with temperature is particularly relevant in vector mosquitoes as immunity is an important determinant of vector competence and pathogen transmission.

Introduction

Immuno-competence is the result of the complex interplay between several immune responses. In vertebrates, this interplay is often between adaptive and innate immunity (176). Although insects have only innate immunity, there is the potential for many interactions between and within their cellular responses – phagocytosis and encapsulation, which are mediated by hemocytes – and humoral responses – soluble effector molecules such as anti-microbial peptides and the enzymes that regulate melanin (177). Part of this interplay is regulatory, for humoral factors do not only contribute to immunity but also control the activity of hemocytes (121). Another part can be a genetic constraint, apparent as genetic correlations between immune components, so that the components cannot evolve independently of each other. The melanization and anti-bacterial responses of the mosquito *Anopheles gambiae*, for example, are positively correlated (178), and the density of hemocytes and the anti-bacterial activity of the Egyptian leaf moth *(Spodoptera littoralis*) and the lytic activity and the encapsulation rate of the Mediterranean Field Cricket *(Gryllus bimaculatus*) are negatively correlated (179,180).

Finally, physiological constraints can impede the activation of an immune response if another response is being activated by a simultaneous challenge. Examples in vertebrates are the interplay between the activation and inhibition of the adaptive immune responses (e.g., (176,181,182) and the upregulation of the humoral response at the expense of the cellular response (182–184). In insects, however, no studies have to our knowledge been conducted on the possible interaction between branches of the innate immune system.

We studied whether two branches of the immune system of the mosquito *Aedes aegypti* – the humoral melanization response and the principally cellular anti-bacterial response – interact with each other. Melanization relies on the aggregation of hemocytes to form a capsule around a foreign body. A cascade of biochemical reactions activates the phenol oxidase cascade (121,166), leading to the deposition of melanin and hardening of the capsule (151). The ability to encapsulate abiotic material has previously been related to the capacity of an individual to clear or limit dissemination of many pathogens including malaria parasites and arboviruses (121,149,150). The anti-bacterial response can involve several biochemical pathways involving the production of anti-microbial peptides such as defensin, the use of oxygen-reactive speciess and phagocytosis (121,152). Which pathway dominates the immune response depends on the insect and the immune stimulant, for example the most dominant pathway of the immune response of *Aedes aegypti* against *E. coli* is phagocytosis (152).

Since the immune responses of insects are strongly influenced by the environment, for example by diet (185), by predator-induced stress (186) or by temperature (187), we also studied how the

melanization and anti-bacterial responses and, in particular, the interplay between them were influenced by temperature.

Materials and Methods

Experimental design

As the phenotypic result of the two immune responses – melanization and anti-bacterial response – cannot be measured in the same mosquito, we measured their interplay in two experiments. In one, we measured the melanization response with or without having stimulated the anti-bacterial response; in the other we measured the anti-bacterial response with or without having stimulated the melanization response. Each experiment was performed at four temperatures, whereby the larvae and adults were maintained at the same temperature and repeated four times in separate experimental blocks.

Mosquito rearing

We used the UGAL strain of *Aedes aegypti,* which was established in 1970 (134) and our laboratory has been maintaining at 26° ± 0.5 °C, 70% ± 10% and 12h:12h light:dark cycle since 2012. *Aedes aegypti* is a vector of several arboviruses, such as dengue, Zika and chikungunya, and of other microparasites, such as the chicken malaria parasite, *Plasmodium gallinaceum* (188). It is found in urban settings throughout the tropics and subtropics (189).

Eggs were soaked in ddH_20 and hatched in a vacuum desiccator for 30 minutes. Six hours after hatching, larvae were moved haphazardly to 12-well plates and kept individually in 3ml of deionized water. Nine 12-well plates were moved to each of four climate-controlled chambers set to 24°C, 26°C, 28°C or 30°C, and to 70% ± 10% relative humidity, and a 12h:12h light: dark cycle.

Every 24 hours, larvae were fed with Tetramin Baby® fish food according to the standard of our laboratory (0-day old: 0.06mg/larva, 1-day old: 0.08mg/larva, 2-day old: 0.16mg/larva, 3-day old: 0.32mg/larva, 4-day old: 0.64mg/larva, 5-day old and older ones: 0.32mg/larva). Pupae were moved to 50ml Falcon™ tubes containing 10ml deionized water, which were covered with mosquito netting. Cotton wool moistened with 6% sugar solution was placed on top of each netting and changed every 24 hours. After emergence males were discarded and 50 females per temperature treatment were kept for an additional four days before injection at their rearing temperature.

Immune assays

Melanization response

The melanization response was evaluated as the degree to which negatively charged CM-25 Sephadex beads (Sigma Aldrich, Steinheim, Germany) were melanized after intrathoracic inoculation (155). The beads range from 50 to 130µm in diameter; we chose the smallest ones by visual inspection. Each day between five and eight days after emergence, we moved 20 mosquitoes per temperature to an empty 20ml Falcon™ tube and immobilized them by placing the tubes on ice for five-ten minutes. We then injected one bead together with either 0.2µl of Luria-Bertani (LB) medium or 0.2µl of LB containing 3500 bacteria (see below) into the thorax of each mosquito using a glass capillary. After injection, the mosquitoes were moved to a plastic cup lined with filter paper, giving the mosquitoes access to 6% sugar solution, and covered with mosquito netting. The mosquitoes were kept at their rearing temperature for 48 hours. The surviving mosquitoes were frozen until further manipulation. Dead mosquitoes were counted but discarded without any further analysis. This entire procedure was repeated four times using four mosquito batches over four days.

We dissected the frozen mosquitoes in 0.1% methyl green colored solution, retrieved the beads, and photographed them under a light microscope with 20x magnification. We estimated the amount of melanin deposited on each recovered bead using ImageJ version 2.3.0 (156) by filtering out the color spectrum of unmelanized beads and measuring the grey values of the beads. We scaled the grey value to give values between zero for unmelanized beads and one for the most strongly melanized beads. To take into account potential confounding factors we also estimated the size of the bead by measuring its diameter with ImageJ and measured the length of wings as an indication of body size from the tip of the wing to the distal end of the alula using ImageJ.

Anti-bacterial response

The anti-bacterial response was measured as the growth within mosquitoes of GFP-expressing *Escherichia coli* strain CIP BZB1011 (190). To find the injection doses we used a previously established standard curve based on the absorbance, measured at a wavelength of 600nm of known concentrations of *E. coli*. We inoculated each mosquito with 3500 bacteria, which had been cultured overnight in Luria-Bertani's (LB) rich nutrient medium in a shaking incubator at 37 °C, and then serially diluted the solution to obtain the desired number in 0.2ul of injection liquid. To prevent further bacterial growth, we put the solution on ice immediately after preparation and up to being injected. A fresh solution was prepared each injection day.

Five to eight days after emergence, we chilled 20 females from each temperature on ice for five to ten minutes and then injected the bacteria into their thorax with a glass microcapillary filled with 0.2 µl of

the bacteria solution together with either a glass bead (which is immunologically inert (191) or a negatively charged Sephadex bead. Similarly sized glass and Sephadex bead were chosen (about 50 to 130µm diameter). This entire procedure was repeated four times using four mosquito batches over four days. The mosquitoes were then moved to plastic cups lined with filter paper covered with mosquito netting and provided access to a 6% sugar solution. We kept them at their rearing temperature for 48 hours and measured the bacterial load in the surviving individuals (80). Briefly, we transferred the mosquitoes into two ml Eppendorf ™ tubes and crushed them in 200µL of Luria-Bertani broth with a micro-pestle. The homogenate was diluted 50- and 200-fold. 50µL of each dilution were spread on separate LB-agar plates. The agar plates were incubated overnight at 37°C and GFP-bacteria colonies were counted under a fluorescent binocular (Discovery. V8, Zeiss). We used the number of bacteria as an inverse measure of the anti-bacterial response.

Statistical analysis

All analyses were done with R for MacOS version 1.4.10 (157). We analyzed the experiments for the two immune responses separately. Both analyses included the competing immune challenge (beads when analyzing the anti-bacterial response, and bacteria when analyzing the melanization response) as a factor with two levels (with or without competing challenge), temperature as a factor with four levels (24°C, 26°C, 28°C and 30°C), their interaction and the experimental block as a factor with four levels (A, B, C, D). The significance for each factor was calculated with the *Anova* function of the *car* package, version 30-10 (192). When the interaction was significant, a type III *Anova* was used, otherwise a type II *Anova* was used. When an effect or an interaction was significant, we used the *emmeans* (Estimated Marginal Means) and *pairs* function of the *emmeans* package, version 1.7.2 (159) for contrast analysis.

Melanization response

The amount of melanin deposited by females injected with a Sephadex bead in the presence or absence of bacteria was tested with a linear mixed effect model assuming a normal distribution of errors. To achieve normality of the residuals, we used the square-root of the scaled grey value to the power five. Normality was visually checked with a *qqplot* and homoscedasticity was tested with a *Breusch-Pagan* test (*bptest* function of the *lmtest* library in R, version 0.9 3 8 (160). In addition to the factors mentioned above, we included wing length and bead size as co-variables.

Anti-bacterial response

Bacterial load was bimodal (see Figure 4). We therefore divided the data into two categories (low and high bacterial density) that were separated by the minimal bacterial count, and we analyzed the data with a generalized linear model (GLM) with a binomial distribution.

Within each category of bacterial load (low vs. high load) we then analyzed the data with a GLM with a quasi-Poisson distribution. So that the data followed the distribution, we used the square-root of the bacterial load in the low-bacteria category and the square of the bacterial load in the high-bacteria category.

Results

We reared 432 larvae at each temperature and inoculated eight to ten females for each challenge at each combination of treatments (at each of the four temperatures, on each of four days, and together with or without the competing challenge).

Melanization response

We inoculated 320 females, nine (2.8%) of which died in the first 48 hours after the injection and were therefore excluded from the melanization assay. Mortality was related to neither immune challenge nor temperature. The injected bead was recovered in 302 of the surviving 311 females.

Every mosquito melanized its bead at least partially. The grey value of the beads differed among temperatures ($F = 3.79$, $df = 3$, $p = 0.011$) (**Figure 1**). It was higher at lower temperature (24°C: 0.66 ± 0.022; 26°: 0.64 ± 0.02) (Mean ± 95% CI) than at higher ones (28°C: 0.58 ± 0.024; 30°C: 0.59 ± 0.023). Melanization was also higher with a competing bacterial challenge (without challenge: 0.61 ± 0.017; with challenge: 0.65 ± 0.016, $F = 9.52$, $df = 1$, $p = 0.0022$). Finally, the grey value of the bead was influenced by the interaction between the temperature and the immune challenge ($F = 3.41$, $df = 3$, $p = 0.017$) (**Figure 1**). Thus, when a bead was injected together with bacteria, the grey value of the beads was higher at 24°C than at any other temperature (**Table 1**), whereas without a bacterial challenge the only observed difference was between 26°C, where the degree of melanization was the highest, and 28°C, where melanization was the lowest (**Table1**). Grey value of the beads was at intermediate levels when tested at 24°C and 30°C (**Table 1**).

Neither bead size $F = 2.91$, $df = 1$, $p = 0.089$) nor wing length ($F = 0.69$, $df = 1$, $p = 0.405$) significantly affected the grey value of the bead but experimental block was significant ($F = 5.28$, $df = 3$, $p = 0.002$).

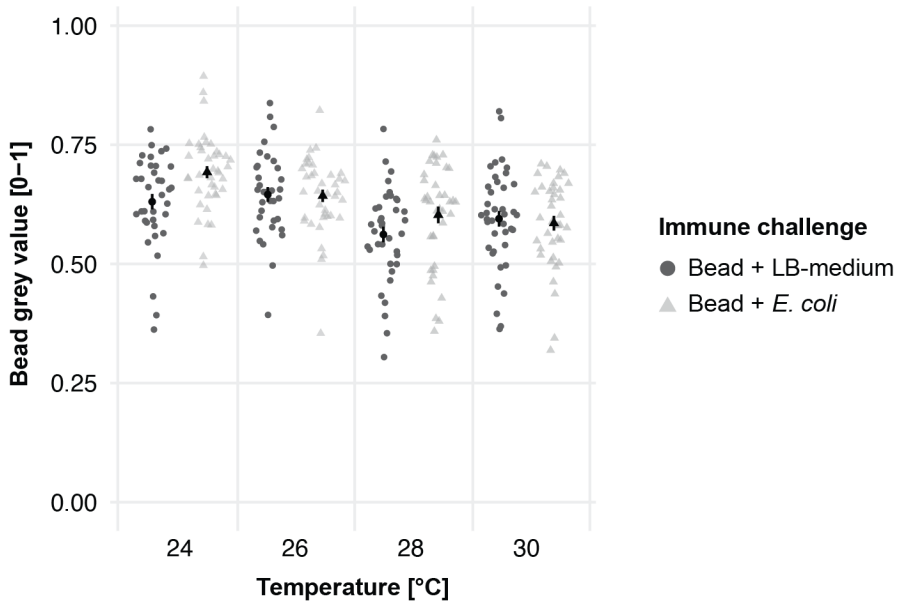

Figure 1: Illustrated here is the amount of melanin deposited on beads 48 hours after being injected into adult *Aedes aegypti* females either in presence or absence of bacteria and at four temperatures (24°C, 26°C, 28°C and 30°C). The mosquitoes were inoculated four days after emergence and beads recovered 48 hours after the injection. The grey value was rescaled so that "0 "means that the bead is white, while a grey value of "1 "means that the bead is entirely black. The sample sizes are from left to right: $N_{24°C}$ = 36 and 40 $N_{26°C}$ = 34 and 39, $N_{28°C}$ = 37 and 38, $N_{30°C}$ = 40 and 38. Error bars show the standard error of the means.

Table 2 Results of the post-hoc tests (Tukey) that compares grey values of the beads (Measure of melanization) 48 hours post challenge at four different experimental temperatures in presence or absence of bacteria. Significance is marked in bold and with an asterix.

		Challenge					
		Bead + LB			*Bead + E. coli*		
		t. ratio	*df*	*p value*	*t. ratio*	*df*	*p value*
Temperature[°C]	24-26	-0.873	287	0.8189	2.634	287	**0.0439***
	24-28	2.336	287	0.0923	3.728	287	**0.0013***
	24-30	0.965	287	0.7696	4.559	287	**<.0001***
	26-28	3.317	287	**0.0056***	1.368	287	0.5203
	26-30	1.871	287	0.2427	2.307	287	0.0988
	28-30	-1.547	287	0.4106	0.996	287	0.752

Anti-bacterial response

We inoculated 313 mosquitoes with *E.* coli. At 24°C, 26°C and 28°C we inoculated 40 mosquitoes for each competing immune challenge (glass bead or Sephadex bead); at 30°C we injected 38 mosquitoes with bacteria and a glass bead and 35 mosquitoes with bacteria and a Sephadex bead. Of the mosquitoes inoculated with a glass bead one died at 24°C, one at 26°C, one at 28°C and three at 30°C; and of the mosquitoes inoculated with a Sephadex bead two, three, two, and three died at the four respective temperatures.

The mosquitoes were more likely to harbor a high bacteria load, if the injection of bacteria was accompanied with a Sephadex bead (76%; 95% ci 69%- 83%) than with a glass bead (53%; 95% ci: 44%-61%; $\chi^2 = 19.5739$, df = 1, $p < 0.001$) (**Figure 2**). While temperature had little impact on bacterial control ($\chi^2 = 5.1377$, df = 3, p = 0.16), the interaction between temperature and competing immune challenge was close to significant ($\chi^2 = 6.6764$, df = 3, p = 0.082) with the strongest effect of the immune challenge at 26°C (90%; 95% ci: 75%-96% vs. 46 %; 95% ci: 30%-62 %) and the weakest effect at 24°C (77%; 95% ci: 11%-17% vs 66%; 95% ci: 14%-17%). Bacterial load was similar among experimental blocks ($\chi^2 = 5.4848$, df = 3, p = 0.13).

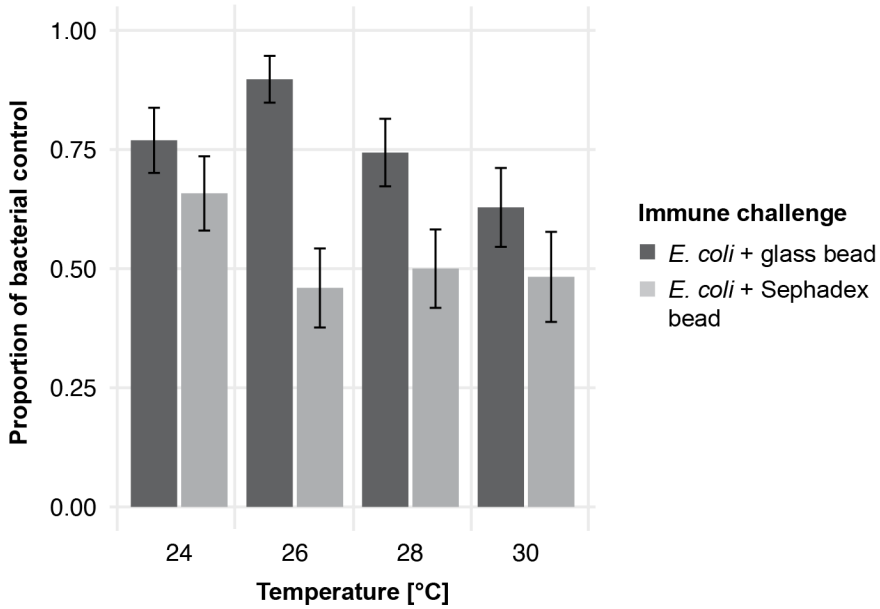

Figure 2: Here are displayed the proportions of individual that at least partially controlled bacterial growth 48 hours after the injection according to temperature (24°, 26°, 28° and 30°C) and co-stimulation of the melanization response or not. Bacterial load was assessed by spreading of the mosquitoes on LB-agar plate and counting of the Colony Forming Units (CFUs) 48 hours after the injection. As a proxy for anti-bacterial response, the lower the bacterial load the higher the specific immune response. Error bars show standard error to the mean.

Within the 191 mosquitoes in the low-bacteria category, the bacterial load was higher in mosquitoes inoculated with a Sephadex bead ($41x10^3$; 95% ci: $25x10^3$ to $58x10^3$) than in those inoculated with a

glass bead ($29x10^3$; 95% ci: $15x10^3$-$43x10^3$; $\chi^2 = 4.65$, df = 1, p = 0.031), whereas neither temperature ($\chi^2 = 1.8616$, df = 3, p = 0.6016) nor the interaction between temperature and bead type ($\chi^2 = 2.6557$, df = 3, p = 0.4478) affected bacterial growth (**Figure 3a**). In contrast, within the mosquitoes in the high-bacteria category, bacterial load increased with temperature ($\chi^2 = 39.05$, df = 3, p < 0.001) (**Figure 3b**). While overall it was not influenced by the type of immune challenge ($\chi^2 = 0.848$, df = 1, p = 0.357), it was affected by an interaction between immune challenge and temperature ($\chi^2 = 9.55$, df = 3, p = 0.023): the difference between the bacterial load of mosquitoes inoculated with a Sephadex bead or a glass bead was highest at 26°C (glass vs Sephadex bead : z = - 2.012, p = 0.044) , intermediate at 24°C (glass vs Sephadex bead: z = - 0.863, p = 0.388) and 28°C (glass vs Sephadex bead : z = -1.076, p = 0.2819) and lowest at 30°C (glass vs Sephadex bead: z = 0.932 , p = 0.3512)(**Figure 3b**).

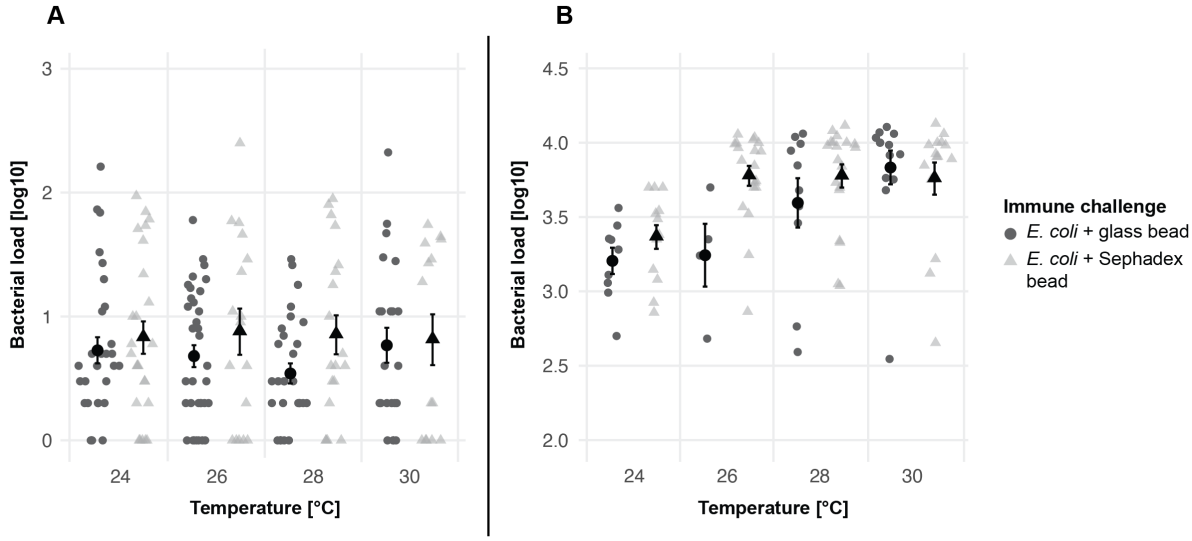

Figure 3: Bacterial load in adult *Aedes aegypti* 48 hours after being inoculated with either GFP- *E. coli* bacteria with a neutral glass bead or with a negatively charged Sephadex bead. In A. is illustrated the number of bacteria (log10 transformed) retrieved at each temperature (24°, 26°, 28° and 30°C) in mosquitoes that displayed an effective control of bacterial growth 48 hours after inoculation. The sample sizes are from left to right: $N_{24°C}$ = 30 and 25, $N_{26°C}$ = 35 and 17, $N_{28°C}$ = 29 and 19, $N_{30°C}$ = 22 and 14). In B. is shown the number of bacteria (log10 transformed) retrieved at each temperature (24°, 26°, 28° and 30°C) in mosquitoes where bacteria grew un-controlled for 48 hours after injection. The sample sizes are from left to right: $N_{24°C}$ = 9 and 13, $N_{26°C}$ = 4 and 10, $N_{28°C}$ = 19 and 19, $N_{30°C}$ = 13 and 18). Error bars show the standard error of the means.

Discussion

Although we had expected that the melanization and anti-bacterial response in mosquitoes would impede each other (178), this was only true for the anti-bacterial response. The anti-bacterial response was hindered by the simultaneous stimulation of the melanization response. We observed the opposite effect on the melanization response which was enhanced by the simultaneous stimulation of the anti-bacterial response. Furthermore, temperature modulated the strength and

interplay between those two branches of the mosquito's immune system, highlighting the complex interaction between immune functions and environmental parameters.

A common assumption is that immune responses are costly, as evidenced by several trade-offs between immunity and other life-history traits (e.g., (175,179,180,193–196). Assuming that resource allocation for immunity is limited, inhibiting one response to the profit of another response might be a physiological constraint of the immune system. We found that to only be the case for the anti-bacterial response. The proportion of individuals that showed some level of bacterial control was lower when the melanization response was simultaneously triggered. However, when faced with a simultaneous melanization and bacterial challenge, mosquitoes exhibited higher level of melanization. This is possibly due to shared pathways of activation and activity between the two responses. In *Aedes aegypti*, the toll pathway involved in bacterial recognition also controls the expression of pro-phenol oxidase genes that are involved in the production of phenol oxidase, which is a key enzyme in the synthesis of melanin (197) . Additionally, within the immune reactions of *Aedes aegypti* in response to *E. coli*, defensin - an important anti-microbial peptide - and phenol oxidase are found together at the locations where melanin is being deposited, and they frequently coexist within the melanotic capsules (198). This suggests that the anti-microbial peptide defensin is involved in the mosquito's melanization response. While melanization is involved in the early initial response against bacteria (152,199), full bacterial control requires the production of anti-microbial peptides (167). Whether bacterial control was hindered directly, indirectly or a combination of both by the melanization response is unclear. Overloading the immune system of *Anopheles gambiae* with multiple Sephadex beads resulted in a non-uniform distribution of melanization among the beads - one bead was prioritized to the detriment of the others (200). A similar pattern where melanization of the bead was favoured over melanization of bacteria which limits early bacterial control might be at play in our experiment. Alternatively, the strong activation of the melanization pathway might directly limit the production of anti-microbial peptides. Measuring immune effectors such as phenol oxidase and anti-microbial peptides and haemocytes activity at different time-point after the individual and simultaneous challenges could help understand the mechanism involved in the interplay between melanization and anti-bacterial responses.

The efficacy of and interplay between immune responses depends on environmental factors. Indeed, in our experiment the melanization response was stronger at lower than at higher temperatures, corroborating studies on *Anopheles gambiae* (162) and *Anopheles stephensi* (104).This effect was especially strong when mosquitoes were challenged in combination with bacteria. One possible explanation is that mosquitoes reared at lower temperature grow more slowly and develop into bigger adults with higher nutrient reserves at emergence (95,201) that express a stronger melanization

response. It is however unlikely to be the main explanation here, as in our analysis body size did not influence the degree of melanization. Another possibility is that since the production of melanin is involved in other physiological functions beyond immunity, for instance egg hardening (163–166), it contributed to the observed faster rate of the humoral melanization response at colder temperature (104). Alternatively, temperature may directly affect immune functions. Indeed, several recent studies on mosquitoes have reported influences of ambient temperature on the expression of many genes, including immune genes (106,202), which may impair immune responses at higher temperatures. However, temperature-dependent changes of immune function in mosquitoes are complex. Different branches of the immune system respond differently to temperature so that at a specific temperature some immunes functions are upregulated, and other functions are downregulated (103). In contrast to the melanization response, the ability of mosquitoes to control bacterial growth was not affected by temperature. Temperature only influenced bacterial load in individuals unable to control the infection (high bacterial load). However, this is possibly reflective of a phenotype of the bacteria itself rather than an effect on the anti-bacterial response of the mosquito. Since *E. coli* has a temperature-dependant growth rate (203,204), increase in bacterial load with experimental temperature can be expected in the absence of bacterial control.

Our results once again highlight the multifaceted aspect of mosquito's immunity. Gaining better understanding of the interplay between immune responses in variable environment may help to understand the evolution of resistance and transmission of disease.

Chapter 3

Thermal adaptation in a major vector species: Adaptation to temperature impacts the thermal response of the yellow fever mosquito

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Abstract

As nearly all aspects of mosquito biology are impacted by temperature, climate change might lead to substantial shifts in the distribution and dynamics of mosquitoes and consequently of mosquito-borne diseases. But climate warming is a long-term process that might give mosquitoes the possibility to adapt to changing temperatures. Although evolutionary thermal adaptation is likely to have a significant impact on the future distribution of vectors and vector-borne disease, it remains largely unexplored.

We addressed the potential for evolutionary thermal adaptation of the yellow fever mosquito *Aedes aegypti* by allowing mosquitoes to adapt to four different temperatures (24°C, 26°C, 28°C, and 30°C) during 16 generations. We assessed the thermal response of these lines at seven different temperatures ranging from 24°C to 34°C by measuring several life-history traits. We found distinct thermal responses between our adaptation lines. The traits that were the most affected were larval development time, adult lifespan, and adult body size. In particular, adaptation to higher temperature resulted in a higher tolerance at high temperatures. Our results suggest that evolutionary adaptation is a likely mechanism that will enable mosquito population to persist in their current geographical range despite climate warming.

Introduction

Vector-borne diseases currently threaten half of the world's population and cause about 500 million cases and one million deaths every year (7). Climate change may make this situation worse, for it is expected to alter the transmission dynamics, geographical distribution, re-emergence, and burden of vector-borne diseases (25). Temperature, for example, constrains the distribution of vector-borne diseases, in part because of thermal limits beyond which the survival of the vectors and the parasites they transmit are inhibited (34,115).

The impacts of increasing temperature on the distribution and intensity of vector-borne diseases are usually studied either by forecasting future outcomes from the current spatial correlations between temperature (and other climatic variables), and diseases (12,14,60,205,206) or with laboratory experiments on the impact of temperature on epidemiologically relevant traits like the longevity of vectors or the developmental period of the parasites (reviewed in (32,207)) and using this information in epidemiological models of the dynamics of vector-borne diseases (reviewed in (208)). Such studies show that in mosquitoes and other ectotherms temperature has strong, non-linear effects on traits such as survival and fecundity (42), with peak thermal performance typically at a temperature close to the upper thermal limit (38–40). Consequently, even small shifts toward warmer temperatures can have drastic effects on their performance as disease vectors and increase transmission (37).

Most of these studies rely on short-term experiments that measure the traits of vectors from a standard colony but reared at different temperatures. But climate change is a long-term process that gives insects – with their short generation times, high population growth rates and strongly temperature dependent traits – time to adapt to their new climatic situation (117,118,120). Rather than relying on the short-term experiments, we should understand this adaptive response to rising temperature to make biologically relevant predictions about the impact of climate change. Such adaptive responses could include shifts of the thermal limits or of the thermal optima or changes of tolerance for heat or cold spikes (32,119).

We addressed whether short-term responses of life-history traits to temperature can give reliable information about the long-term, evolutionary responses to climate change, focusing on the longevity of mosquitoes. Longevity is a central epidemiological trait in the epidemiology of vector-borne diseases, for the parasite can only be transmitted, if the mosquito lives long enough for the parasite to complete its development and produce infectious stages. Temperature has a large impact on longevity. In particular, mosquitoes live longest at an intermediate temperature, and live less long as temperature moves away from this optimal value, so that warmer climates can be expected to either increase or decrease the probability that mosquitoes transmit diseases (12).

The main aim of our experiment was to see whether the impact of temperature on longevity of mosquitoes is affected by adaptation to warmer or colder temperatures. We therefore let the mosquito *Aedes aegypti* adapt to 24°C, 26°C, 28°C, and 30°C for 16 generations. We chose these temperatures, for they are close to the average temperature typically experienced by *Aedes aegypti.* We then measured how their longevity (and other life-traits: larval mortality, larval development time and adult size) responded to being reared at environmental temperatures ranging from 24°C to 34°C.

Materials and Methods

The mosquitoes used for this experiment originated from a colony of the UGAL strain of *Aedes aegypti t*hat was established in the 1970s (134). We obtained the colony from Patrick Guérin (Université de Neuchâtel) and have been maintaining it for 10 years under standard insectary conditions (26.5 ± 0.5°C, 75 ± 5 % humidity, 12:12 hours dark:light cycle).

Experimental evolution

We let three lines of mosquitoes adapt to each of four temperatures – 23.9°C, 26°C, 28.1°C, and 30.2°C. For simplicity, we will refer to these adaptation temperatures as 24, 26, 28, and 30°C. We started the evolution with larvae from the colony that had been maintained at 26.5°C, reared this cohort at 26°C, then increased or decreased the temperature by 0.7°C every generation until the target temperature was reached, and maintained each line at its target temperature for at least 10 generations.

Every generation, dried eggs from the previous generation were rehydrated in deionized water for 30 minutes and hatched in a reduced-pressure desiccator. Six hours after hatching, 400 first instar larvae from each line were moved to two plastic trays (35 x 21 x 4 cm) containing 800ml deionized water. Larvae were fed daily with Tetramin Baby® fish food according to their age: 0.06, 0.08, 0.16, 0.32, 0.64, 0.32 mg per larva for age 0, 1, 2, 3, 4, and 5 days or older (61). Pupae were moved to 300ml plastic cups containing 100ml of water, which were placed into a 21 x 21 x 21cm, plexiglass cage (one cage per line) for emergence. After emergence, the cups were removed from the cages. Adults had constant access to a 6% sucrose solution. They were given the opportunity to blood-feed on AK's arms for seven minutes once early and once late in life. That is 17 and 31 days after the eggs were hatched (so when adults were four to seven days old and 16 to 20 days old). Two days after the blood-meals, egg-laying dishes (300ml plastic cups containing 100ml deionized water and lined with filter paper) were put into the cages. Three days later, the filter papers were removed, dried at room temperature, and kept in dark storage at room temperature until being used for the next generation.

Measuring impact of adaptation

To assess how adaptation affects the response of mosquitoes to temperature, we measured several life history traits – larval mortality, larval development time, adult body size and adult longevity – of the adapted mosquitoes to temperatures ranging from 22°C to 34°C. Because only four temperature-controlled chambers were available, the experiments were run in two blocks, one at rearing temperatures 24°C, 26°C, 27°C and 32°C and the other at rearing temperatures 22°C, 28°C, 30°C and 34°C. The first block was run with 5760 mosquitoes, so 120 mosquitoes per line and rearing temperature, and the second was run with 6912 mosquitoes, so 144 mosquitoes per line and temperature. Because of technical issues with the chamber set to 22°C, this temperature will not be considered here.

Eggs were rehydrated in deionized water for 30 minutes and hatched in a reduced-pressure desiccator for 30 minutes. Six hours after hatching, larvae were moved into individual wells of 12-well plates. Each well contained three ml of deionized water to which the daily ration of Tetramin Baby® fish food was added (0.06, 0.08, 0.16, 0.32, 0.64, 0.32 mg for ages 0, 1, 2, 3, 4 and 5 days or older). Pupae were moved to 50 ml Falcon™ tube containing with 10 ml of deionized water and covered with mosquito netting. After emergence, males were discarded and females were moved to individual 120 ml plastic cup lined with filter paper, half-filled with deionized water, and covered with mosquito netting. A petri dish with the same diameter as the cup was placed above the water, so that the mosquitoes could not drown in the water. A piece of cotton wool soaked in 6% sugar was placed on each cup daily. Mosquitoes were checked daily for mortality. Wing length was measured as a proxy for mosquito body size (209). Therefore, the wings of all females were placed onto a glass slide, scanned, and measured from the distal end of the alula to the tip of the wing (vein R3) with ImageJ version 2.3.0 (156).

Statistical analysis

All statistical analyses were done using R version 4.1.10 (157). All analyses included the effect of the experimental temperature as a continuous parameter, adaptation temperature as a factor with four levels, their interaction, and the adaptation line as a random factor nested within adaptation temperature. In the analysis of longevity, the effect of experimental temperature was clearly not linear, so that we scaled the temperatures from 24°C to 34°C to values between 0 and 1 and included the squared values. Significance levels of the tested variables were calculated with the *Anova* function of the *car* package version 30-10 (158); the main effects were tested with a type III *Anova* if the interaction was significant, and with a Type II *Anova* if the interaction was not significant. For continuous variables fit of the residuals to the chosen distribution was checked visually with *qqplots*,

and homoscedasticity was tested with *Breusch-Pagan* tests (*bptest* function of the *lmtest* library R, version 0.9 3 8 (160))

Mortality during the larval development was analyzed with a generalized linear mixed effect model (GLMer) using a binomial distribution of error. Larval development time, that is the number of days between hatching and pupation, was analyzed with a generalized linear mixed effect model (GLMer) using a Poisson distribution of error. Wing length was analyzed with a linear mixed effect model (LMer) using a normal distribution of error. To reach normality of the residuals, we used the squared value of the wing length. Longevity was analyzed with a linear mixed effect model (LMer) with a normal distribution of error. To account for the strong non-linearity of the response to experimental temperature, we included the squared temperature. Nelder-mead optimizer was used to help with the convergence of the model.

Because wing length and longevity are not independent and are both outcomes of the experimental procedure, we also assessed the direct and indirect (via wing length) effects of experimental and adaptation temperature on longevity with a piece-wise structural equation model (pSEM) (210).

Results

Larval mortality

Of the 10 944 mosquitoes reared 5.08% (95% ci: 4.69% -5.52%) died during larval development. Among the adaptation temperatures, larvae were most likely to die at 24°C (5.81% (95% ci: 4.98% - 6.77%)) and among experimental temperatures, larvae were most likely to die at 32°C (10.3% (95% ci: 8.85% -12.1%)), though the effects of adaptation and experimental temperatures were not significant (adaptation temperature: $\chi^2 = 4.42$, df = 3, p = 0.2199; experimental temperature: $\chi^2 = 0.0392$, df = 1, p = 0.8431; interaction; $\chi^2 = 2.8368$, df = 3, p = 0.4175).

Larval development

Developmental time (from hatching to pupation) ranged from 5 to 10 days. It was longest (8.24 days ± 0.03 days) (Mean ± 95% ci) at the coldest experimental temperature and shortest at the warmest temperature (6.14 days ± 0.02 days) ($\chi^2 = 264.58$, df = 1, p <0.0001) (**Figure 1**). While there was no main effect of the adaptation temperature ($\chi^2 = 1.81$, df = 3, p = 0.61), the decrease in developmental time with experimental temperature was stronger in groups adapted to 24°C and 26°C than in groups adapted to 28°C and 30°C (interaction between experimental and adaptation temperature: $\chi^2 = 56.88$, df = 3, p <0.0001).

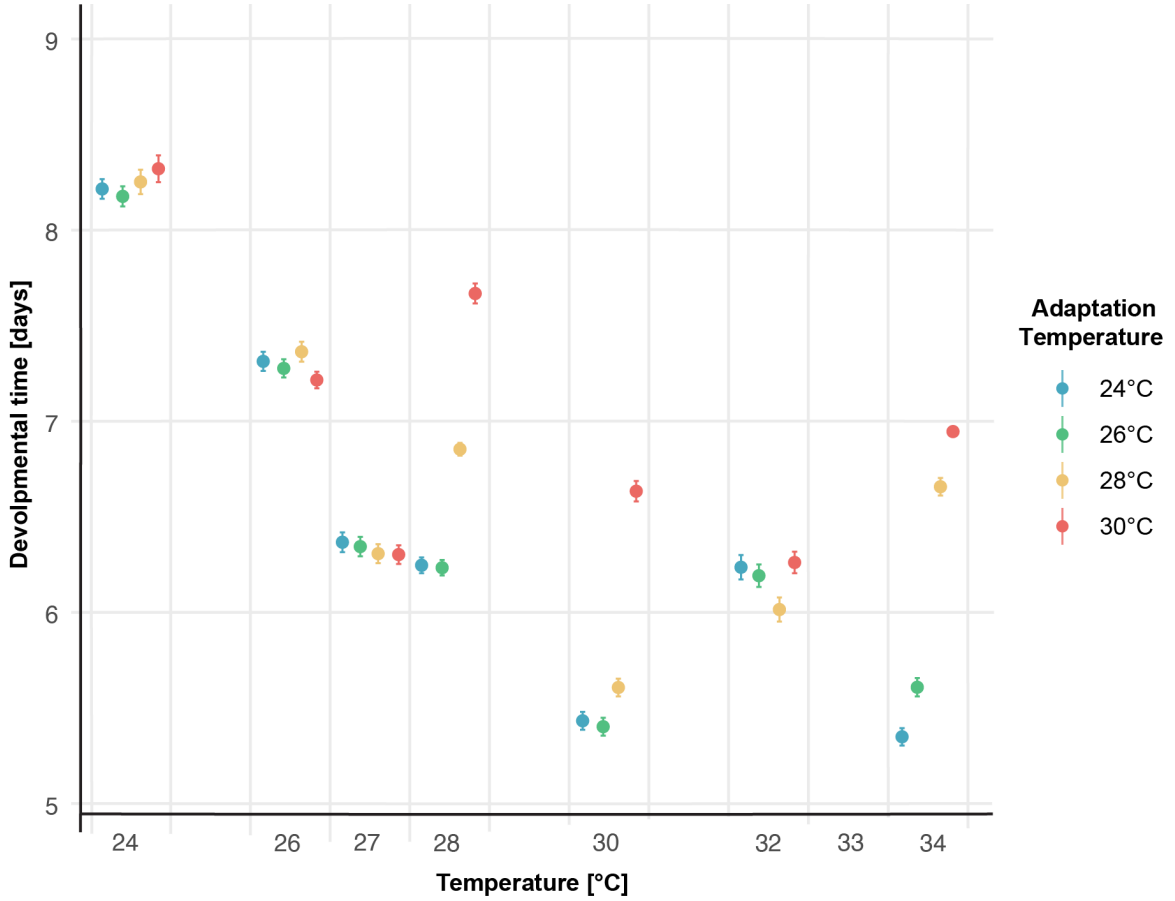

Figure 2: Larval developmental time (from hatching to pupation) in function of experimental temperature. Blue points are individuals previously adapted to 24°C, green points individuals previously adapted to 26°C, yellow points individuals previously adapted to 28°C, red points individuals previously adapted 30°C. Represented are the mean and 95% confidence interval.

Wing length

We were able to measure the wings of 3792 females. Wing length varied between 2.14mm and 3.61mm, with an average of 2.87mm. It decreased with increasing experimental temperature (χ^2 = 656.32, df = 1, p <0.0001) and increasing adaptation temperature (χ^2 = 24.16, df = 3, p <0.0001), but the impact of adaptation temperature was most apparent at experimental temperatures below 28°C (interaction between experimental and adaptation temperature: χ^2 = 21.16, df = 3, p <0.0001) (**Figure 2**).

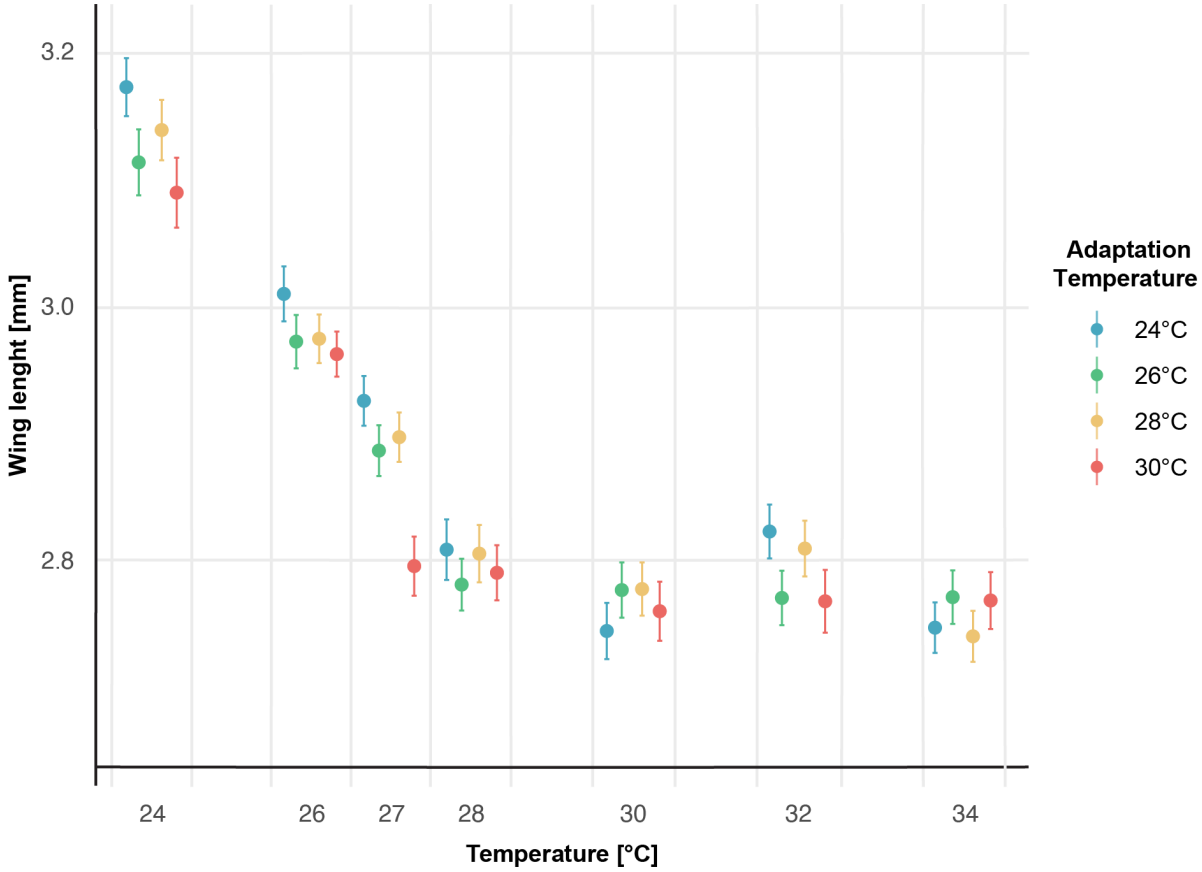

Figure 2: Wing length in mm in function of experimental temperature. Blue points are individuals previously adapted to 24°C, green points individuals previously adapted to 26°C, yellow points individuals previously adapted to 28°C, red points individuals previously adapted 30°C. Represented are the mean and 95% confidence interval.

Longevity

We've monitored the longevity of 3854 females at seven different temperatures. Between 116 and 150 females for each adaptation temperature were tested at each temperature. Longevity ranged from 2 to 60 days with an average of 27 days ± 0.27 days (Mean ± 95% ci).

Longevity was generally longest at intermediate experimental temperatures and lowest at the extreme experimental temperatures (linear effect: $\chi^2 = 15.59$, df = 1, $p < 0.0001$; quadratic effect: $\chi^2 = 29.83$, df = 1, $p < 0.0001$). This pattern was influenced by the adaptation temperature (interaction between adaptation and squared experimental temperature: $\chi^2 = 47.78$, df = 3, $p < 0.0001$. Mosquitoes adapted to 24°C lived a similar time at all experimental temperatures except at 34°C, at which longevity dropped about seven days; longevity was similar for all experimental, those adapted to 30°C lived longest at 28°C and lived similarly long at 24°C and 34°C; those adapted to 26°C or 28°C were in between these patterns. In particular, the mosquitoes adapted to 26°C lived longest at 28°C. (**Figure 3**).

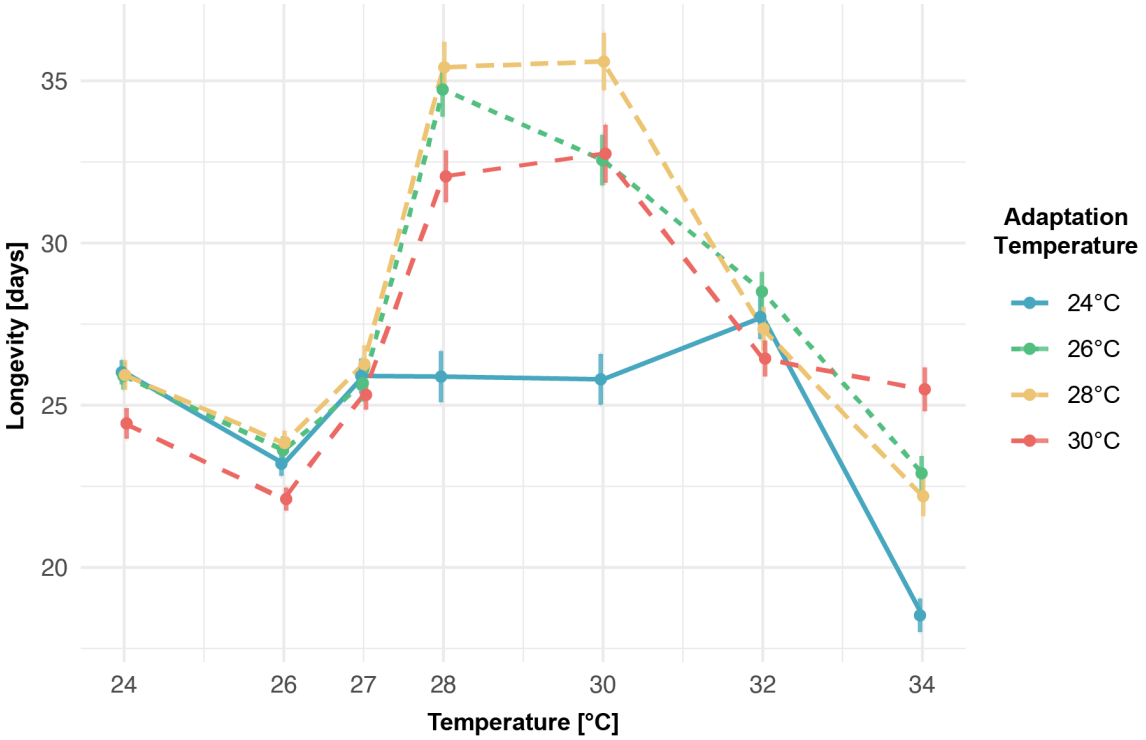

Figure 3: Longevity in days in function of experimental temperature. Each line represents the average longevity of groups of mosquitoes adapted for 18 generation at 24°C (Blue), 26°C (Green), 28°C (Yellow) and 30°C (Red) across seven temperatures. Error bars show the 95% confidence interval around the mean.

Direct and indirect effects

The piecewise structural equation model confirmed that higher experimental and adaptation temperatures led to shorter wings (**Figure 4**). It also showed that the main effect of experimental on longevity are partly due to a direct effect of temperature and partly to an indirect effect; colder temperature leads to larger mosquitoes and larger mosquitoes live longer. In other words, the response of longevity is correlated to changes in wing lengths. The effect of adaptation and of the interaction between experimental and adaptation temperatures on longevity appears to be mainly an indirect effect through the interaction's impact on wing length; both had no significant direct effect on longevity.

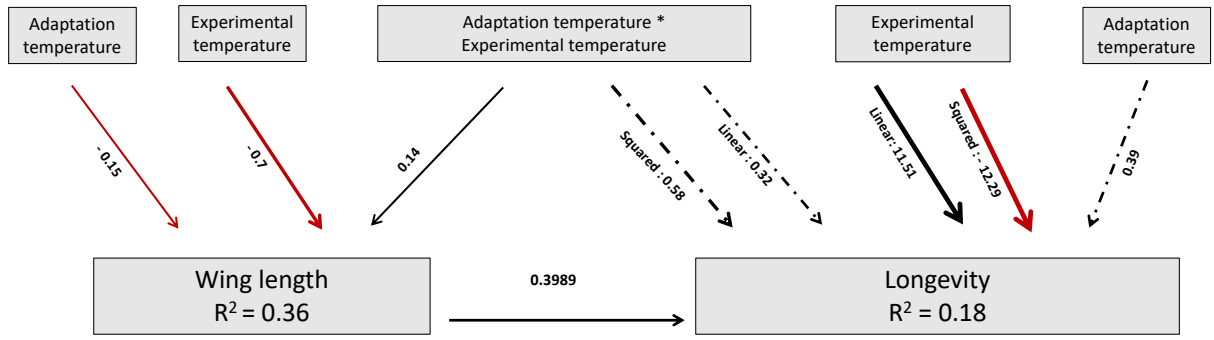

Figure 4: Piecewise structural equation modelling of the interaction between selection temperature, experimental temperature, wing length and longevity. Red arrows show negative correlation and black arrow positive ones. The strength of each relation is indicated by the width of the arrow and its corresponding coefficient. Filled paths are significant and dotted paths non-significant.

Discussion

Evolutionary adaptation is one mechanism enabling mosquito speciess to cope with and persist in a warming climate (119,211–214). In this study, we present indication of evolutionary thermal adaptation in *Aedes aegypti* populations that were maintained at four different temperatures for 16 generations. Overall, the short-term responses echo the findings of numerous studies on the impact of temperature. As in most ectotherms (215), elevated temperatures expedite mosquito development and reduce adult size in mosquitoes (66). However, the scope of these responses varies according to the adaptation temperature. This divergence is evident in larval development, body size and lifespan.

Larvae, following a well-documented trend in response to temperature, pupate more swiftly as temperatures rise (79,216,217). This trend is more pronounced in mosquitoes acclimated to cooler temperatures (24°C and 26°C) compared to those adapted to warmer climates (28°C and 30°C). The longest development duration occurs at 24°C, requiring a minimum of eight days for larvae to transform into pupae, irrespective of their prior selection temperature. Conversely, at the highest rearing temperature of 34°C, larval development accelerates with adaptation temperatures: mosquitoes acclimated to 24°C and 26°C pupate in five days, those from 28°C in six, and those from 30°C in seven. This conforms to the standard seven-days developmental period for *Aedes aegypti* (85), signifying that the 30°C-adapted mosquitoes evolved an enhanced temperature tolerance for this trait.

Adult mosquitoes became smaller with increasing rearing temperatures within physiological limits (218,219). Our mosquitoes were biggest when reared at 24°C and decreased in size linearly with increasing temperatures before being stabilized at rearing temperatures of 28°C and more. Furthermore, differential adaptation to varied temperatures yields dissimilar body sizes within the same rearing temperature. When reared between 24° and 28°C, individuals adapted to 24°C outsized

those from other temperatures, while those adapted to 30°C were the smallest, a pattern potentially due to maternal effects. Larger females tend to engorge on larger blood meals and lay more nutrient-rich, substantial eggs, thereby potentially fostering better-starting conditions for emerging larvae (172,174,220). Altogether, the variance in wing length correlates with the slower development, yielding larger females corroborating the idea that adult size is primarily driven by larval growth and developmental time (90,94,215).

For all adaptation lines expected the cold-adapted ones, longevity peaked at intermediate temperatures and decreased towards the colder and warmer end of the thermal range. This largely corroborates other studies in which adult longevity of *Aedes aegypti* was maximal at around 25°C (32,124). Here, longevity steeply increased and peaked between 28°C and 30°C for lines adapted to 26°C, 28°C and 30°C, whereas lines adapted to 24°C had similar average lifespan between 24°C and 32°C. Moreover, while the adaptation groups at 24°C, 26°C and 28°C all experienced a decrease in averaged longevity at 34°C, suggesting that they are approaching their thermal limit, the ones adapted to 30°C did not. This suggests that our lines exhibit a higher thermal tolerance for longevity than previously reported and that adaptation to higher temperatures gives higher tolerance at high temperatures. However, according to our path analysis, while experimental temperatures both directly and indirectly affected female adult longevity, adaptation temperatures only indirectly - via wing length- influenced the adult lifespan considering the positive correlation between wing length and longevity. This suggests that the correlations among traits – in our case, body size and longevity – also change with the mosquito's adaption.

To summarize, the long-term, evolutionary responses to temperature of several life-history traits largely reflect the short-term responses due to thermal acclimation in a single generation. Thus, for example, starting at a baseline of 26°C, increasing the temperature to 30°C in a single generation or letting the mosquitoes adapt to 30°C both shortened the larval development, decreased adult size, and increased longevity. Quantitative details, however, differed. In particular, how longevity, a main determinant of the intensity of transmission, responded to temperature was strongly affected by the temperature at which the mosquitoes had evolved, so that the temperature that gave peak longevity depended on the adaptation temperature. Therefore, relying on short-term experiments for predictions about the impact of climate change on life-history traits underlying the epidemiology of vector-borne diseases is, to some degree, misleading.

Chapter 4

Temperature sensitive immune functions and vector competence for dengue is unaffected by thermal adaptation in the yellow fever mosquito

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Abstract

Temperature is an important driver of mosquito-borne disease as it strongly impacts physiology and immune profiles of mosquitoes. To date, most studies that investigated the effects of temperature on vector competence in mosquitoes have associated warmer temperatures with impaired immune functions and higher virus transmission rates. This leads to the belief that the burden of vector-borne disease might increase in the face of anthropogenic-driven climate warming. However, these studies do not take into account that mosquitoes can adapt to changing temperatures. We here investigated how evolutionary thermal adaptation impacted the effects of temperature on the immune response and vector competence of *Aedes aegypti*. To do so, we let mosquitoes adapt for more than 18 generations to different temperatures ranging from 24°C to 30°C. Following this, we assessed their anti-bacterial response, as well as their competence for dengue. While increasing environmental temperatures strongly impeded the strength and efficiency of the anti-bacterial response and enhanced the vector competence of *Aedes aegypti* for DENV-1, thermal adaptation did not influence the outcome.

Introduction

The higher temperatures linked to climate change are often expected to shift the distribution of important mosquito-borne diseases such as malaria and dengue, by increasing the risk of transmission as temperate regions, get warmer and by decreasing the risk of transmission in hot regions where the temperature could rise above the optimal values of the mosquitoes and parasites (221).

One of the reasons for such changes is that temperature influences the physiology, and thus the immune response, of cold-blooded animals like mosquitoes. For example, the expression of the immune genes of the mosquito *Aedes aegypti* infected with Zika or chikungunya viruses (105,106), is strongly linked to temperature. It therefore seems clear, that increasing temperature will affect the ability of mosquitoes to transmit pathogens and thus the epidemiology of the diseases they cause (34). Yet, although most studies on the impact of temperature on vector competence have linked warmer temperature to more intense transmission (34), the details of these effects are far from clear.

This is partly because the effects of temperature on mosquitoes are complex and, in particular, differ among aspects of the immune response and other traits. In *Anopheles stephensi*, for example, some immune pathways are upregulated whereas others are downregulated when the temperature increases (104). Indeed, in some mosquito-virus interactions, such as that between chikungunya and *Aedes aegypti*, higher temperatures decrease rather than enhance vector competence (59,105,217,222). It is also partly because the short lifespan of mosquitoes relative to the time over which climate change increases temperatures, giving them ample time to adapt to changing temperatures (117,118,120). Yet, the potential for mosquito thermal adaptation remains poorly investigated, for on the one hand it is generally not incorporated in current prediction models (119) and on the other hand experimental studies generally consider mosquitoes from a colony held over many generations at (that is, adapted to) a constant temperature and then moved for a generation or two to different temperatures (to which they can acclimate) or in which they were collected from the field without considering the population's history.

Here we argue that, if adaptation to rising temperature influences immune responses and vector competence, letting mosquitoes acclimate (within a single cohort) rather than adapt (over several generations) to increasing temperature may give misleading results. We therefore explicitly distinguish between adaptation and acclimation (i.e., rearing and maintenance) of mosquitoes to several temperatures. Thus, we let *Aedes aegypti* adapt during 19 to 20 generations to several temperatures, and then let each line acclimate by rearing one cohort of larvae and adults at the different temperatures. In each of these lines, we then measured the growth of the bacteria *E. coli* (as

a measure of the mosquitoes' immune response and the response to the dengue virus DENV-1 (as a measure of vector competence for dengue).

Materials and Methods

Mosquitoes and parasites

We used the UGAL strain of *Aedes aegypti* (134), a GFP-expressing *E. coli* (strain BZB10B11*)* bacteria (190)- kindly provided to us by Diego Gonzalez (Laboratory for microbiology, University of Neuchatel)-, and the wild-type DENV-1 KDH0026A isolate (223) - GenBank: HG316481.1).

Adaptation

We let three lines of mosquitoes adapt to each of four temperatures – 23.9°C, 26°C, 28.1°C and 30.2°C. For simplicity, we will refer to these adaptation temperatures as 24°C, 26°C, 28°C and 30°C. We began the adaptation experiment by rearing at 26°C one cohort of mosquitoes from our in-house *Aedes aegypti* colony that has been maintained since 2012 at 26.5°C ± 0.5 °C (75 ± 5 % humidity, 12:12 hours dark:light cycle). We divided the progeny of this cohort into twelves groups of which three were assigned to each adaptation temperatures. We subsequently increased or decreased the temperatures by 0.7°C at each generation until we reached the target temperatures. All lines were maintained for at least 13 more generations at their adaptation temperature.

Every generation, we rehydrated desiccated eggs from the prior generation in deionized water for 30 minutes. These eggs were then hatched in a reduced-pressure desiccator. Six hours post-hatching, 400 first instar larvae from each lineage were transferred to separate plastic trays (35 x 21 x 4 cm) containing 800ml of deionized water. The larvae were provided daily nourishment with a predetermined dose of Tetramin Baby® fish food depending on their age: 0.06, 0.08, 0.16, 0.32, 0.64, and 0.32 mg per larva for ages zero, one, two, three, four, and five days and beyond, respectively (61). Pupae were transferred to 300ml plastic cups filled with 100ml of deionized water.

Cups were placed into a plexiglass cages (21 x 21 x 21cm), one cage per lineage, to facilitate emergence. Once emerged, the cups were removed from the cages. Adult mosquitoes were provided continuous access to a 6% sucrose solution. They were given the opportunity for blood-feeding on AK's arms for seven minutes at two distinct time points in their lives: 17- and 31-days following egg hatching, corresponding to ages four to seven days and 16 to 20 days. Two days after the blood meals, we introduced egg-laying dishes, comprised of 300ml plastic cups containing 100ml of deionized water and lined with filter paper, into the cages. Three days later, the filter papers were collected, air-dried at room temperature, and stored in darkness at room temperature for utilization in the next generation.

Anti-bacterial response

General design

After 19 generations we haphazardly selected two lines of each adaptation temperature and acclimated each line to the four temperatures (24°C, 26°C, 28°Cand 30°C) by rearing larvae and adults of each line at each temperature. We then inoculated adult mosquitoes with GFP-expressing *E. coli* and measured the bacterial load in each mosquito two days later.

Mosquito rearing

We hatched eggs by rehydrated them in deionized water for 30 minutes and then placing them into a reduced pressure desiccator for 30 minutes. Since the larval development lasts about eight, seven, six and five days at 24°C, 26°C, 28°C and 30°C, respectively, the hatching was done on separate days, letting adults emerge on similar days.

Six hours after hatching, the larvae from each treatment were transferred to 12-well plates and were fed daily with Tetramin Baby® fish food (0.06, 0.08, 0.16, 0.32, 0.64, 0.32 mg per larvae aged zero, one, two, three, four, and five days or older, respectively). Pupae were transferred to a 50ml Falcon™ tube containing deionized water and covered with mosquito netting. A fresh cotton ball soaked in 6% sugar solution was placed on top of each Falcon™ tube. After emergence the males were discarded, and the females were kept at their respective temperature for another five days. For each treatment, humidity was kept at 70 % ± 10% the photoperiod was 12h:12h light:dark.

Anti-bacterial response

To assay the anti-bacterial response, we measured bacterial growth 48 hours after inoculating five- to six-day old mosquitoes with *E. coli,* following the procedure described in Reitmayer et al 2021 (140). Briefly, 3500 GFP-expressing *E. coli* (strain BZB10B11), together with 0.2µl of Luria-Bertani's (LB) medium were injected into the thoraxes of females. Mosquitoes were then placed into a 120ml plastic cup and kept for 48 hours at their rearing temperature. Surviving mosquitoes were homogenized in 1ml of LB medium and incubated for 6 hours in a shaking incubator at 37°C, 200µl of the homogenate were pipetted in duplicates into a 96-well plate, and the GFP signal expressed by live bacteria was measured by a multimode microplate reader (SpectraMax I3x) at 485/512 excitation/emission. To normalize the plate readouts, we used the fluorescent values of LB medium as a negative control, and we used the fluorescent values of the solution used for the injection as a normalizing standard for each plate.

Statistical analysis

The analyses (done with R version 4.1.10 (157)) included adaptation temperature (as a factor with four levels; 24°C, 26°C, 28°C and 30°C), experimental temperature (as a factor with four levels; 24°C, 26°C, 28°C and 30°C) and their interaction as independent variables. Experimental block was a random factor and lines were nested within their adaptation temperature. Significance for the individual variable and their interaction was calculated with the function *Anova* from the *car* package version 30-10, (158). If the interaction was significant, we used a Type III *Anova*, otherwise we used a Type II *Anova*. We first analyzed bacterial clearance with a generalized linear mixed effect model (function *Glmer* from the *lme4* package version 1.1-32, (224) with binomial distribution of errors. We then analyzed bacterial load for individuals that had not cleared the infection with a linear mixed effect model with a normal distribution of errors (function *Lmer* from the *lme4* package). To reach normality of the residuals we used as dependent variable the transformation log10(bacterial load + 3). Normality of the residuals was visually checked with a *qqplot*, and homoscedasticity was tested using a *Breusch-Pagan* test (function *bptest* function of the *lmtest* library R version 0.9 3 8 (160)). Significant effects of the interaction were further explored with a contrast analysis based on the functions *emmeans* and *pairs* function of the *emmeans* package version 1.7.2 (159). P-values were adjusted with the Tukey method.

Dengue infection

General design

After 20 generations, we reared larvae and adults of each line of three adaptation temperatures (24°C, 28°C and 30 °C) in the three temperatures and offered the females a dengue-infected blood meal. We assessed the infection prevalence, dissemination prevalence and disseminated viral load. The experiment was repeated three times in three separate experimental blocks.

Mosquito rearing

Rearing of mosquitoes was done as described above, except that larvae were kept in 15cm diameter trays containing dechlorinated tap water and adults were kept in 800ml plastic cups and offered a 10% sugar solution.

Dengue infection

Females were infected with dengue virus inside biosafety level-3 containment facilities, following standard methods of the laboratory (225). 40 to 60 five to six days old females were isolated and deprived of sugar 24 hours before the infection and then allowed to feed for 20 minutes through a desalted pig-intestine membrane from a feeder (Hemotek Ltd) set to 37°C. Each feeder contained a

DENV-1 infectious blood meal (two ml of fresh goat blood, 500µl DENV-1 (concentration: 2.5 X 10^7 FFU/ml-Block 1 and 2 and 5.8 X 10^7 FFU/ml – Block 3), 500µl of 2% SVF culture medium and 60µl of ATP 5mM). Fed females were chilled on ice, and engorged individuals were transferred to 500ml paper cups and maintained for 12 days at their rearing temperature with access to 10% solution.

Infection and dissemination prevalence

Twelve days after the infectious blood-meal the presence of viable DENV in either the head (as a measure of disseminated (that is, infectious virus) or the rest of the body (as a measure of infection) was determined in the surviving females following standard procedures (225). Heads were placed into a two ml Eppendorf™ tube containing about 20 1mm-glass beads and stored at -80°C until further manipulation. The rest of the bodies were placed into a 1.2ml Qiagen microtube containing 300µl of squash buffer (Tris 10 mM, NaCl 50 mM, EDTA 1.27 mM with a pH adjusted to 9.2 and supplemented with proteinase K at a concentration of 0.35mg/L) (Eurobio Scientific). The bodies were homogenized with a Qiagen Tissuelyzer™ at 30 cycles/s for 1 minute and centrifuged at 17,000 g for 5 minutes at 4° C. 100 µl of the supernatant were transferred into 96- well plates and incubated for 5 minutes at 56 °C, followed by 10 minutes at 98°C for RNA extraction. Viral RNA was detected with a two-step RT-PCR using DENV-1-specific primers that target a region of the NS4 gene: F: 5': -CAGGACAACCAGCTGGCATA – 3', R: 5'-CCAAGGCGAGAAGTGGAACT – 3' and producing a 355 bp fragment. Total RNA was reverse transcribed into cDNA with M-MLV reverse transcriptase (ThermoFisher Scientific) and random hexameric primers. The DNA was amplified with DreamTaq™ DNA polymerase (Thermofisher Scientific). Amplicons were visualized by electrophoresis on a 1.5% agarose gel. The settings of the thermocycler for the reverse transcriptase and PCR can be found in Fontaine et al (225).

Disseminated viral load

We measured the disseminated viral load with a standard focus-forming assay (FFA) in C6/36 mosquito cells (225). Each head was homogenized in 300ml of Leibovitz's L-15 medium supplemented with 2x antibiotic-antimycotic (Life Technologies). The wells of 96-well plates were seeded with convergent C6/36 cells, inoculated with 40µl of the homogenate and incubated at 28°C for one hour. The cells were then overlaid with a 1:1 mixture of 3.2 % carboxymethyl cellulose (CMC) and Leibovitz's L-15 medium supplemented with 1% penicillin / streptomycin, 2% Tryptose Phosphate Broth ,1x 1% nonessential amino acids, 2× Antibiotic-Antimycotic (Life Technologies), and 10% FBS. The cells were incubated at 28°C for three days and then fixed with 3.7% formaldehyde, washed three times with PBS and further incubated with 0.5% Triton X-100 in PBS at 28°C for one hour. They were further incubated at 37°C for one hour with a mouse anti-dengue monoclonal antibody (MAB8705, Merck

Millipore) and washed three times with PBS. Finally, they were further incubated for 30 minutes with an Alexa Fluor 488-conjugated goat anti-mouse antibody (Life Technologies).

Each female was deposited pure, 10x and 100x diluted. We then counted the infectious foci under a fluorescent microscope. The mean value of the two highest countable units were converted into focus-forming units/mosquito (FFU/head).

Statistical analysis

All analyses were done with R version 4.1.10 (157) and included as explanatory variables the adaptation temperature (as a factor with three levels; 24°C, 28°C and 30°C), the experimental temperature (as a factor with three levels; 24°C, 28°C and 30°C) and their interaction. Experimental blocks and lines nested within their adaptation temperature were considered as random factors. To calculate significance of each variable, we used the function *Anova* from the *car* package version 30-10, (158). When the interaction revealed to be significant, we used a type III *Anova*; otherwise, we used a Type II. Whenever relevant, the functions *emmeans* and *pairs* from the *emmeans* package version 1.7.2 (159) were used for post-hoc analysis. P-values were adjusted with the Tukey method.

Infection prevalence (i.e., the proportion of bodies that were infected) and dissemination prevalence (i.e., the proportion of heads that were infected) were analyzed with generalized-linear mixed effect models (function *glmer* from the *lme4* package version 1.1-32, (224)) using a binomial distribution of errors. The disseminated viral load (i.e., log10 transformation of DENV-1 focus-forming units/head) was analyzed with a linear mixed effect model (function *lmer* of the *lme4* package). Normality of the residuals was visually assessed with a *qqplot*, and homoscedasticity was tested with a *Breusch-Pagan* test using the function *bptest* of the *lmtest* package version 0.9 3 8 (160).

Results

Antibacterial response

We inoculated 1019 mosquitoes, of which 19 died within 48 hours of the inoculation and were not further considered in the analysis. We tested 267 individuals that had been reared at 24°C, 236 at 26°C, 256 at 28°C and 260 at 30°C. 31.5 % (95 % ci: 28.64 % to 34.5 %) of the mosquitoes cleared the bacterial infection within 48 hours (**Figure 1**). Neither adaptation temperature ($\chi^2 = 0.91$, df = 3, p = 0.823) nor the interaction between adaptation and experimental temperatures ($\chi^2 = 5.73$, df = 9, p = 0.766) were linked to the clearance rate, but experimental temperature was ($\chi^2 = 16.82$, df = 3, p = 0.001). Mosquitoes were much more likely to clear the infection if they had been reared at 24°C than if they had been reared at a warmer temperature (pairwise comparisons 24°C vs other temperatures: $p < 0.024$; other comparisons: $p > 0.86$).

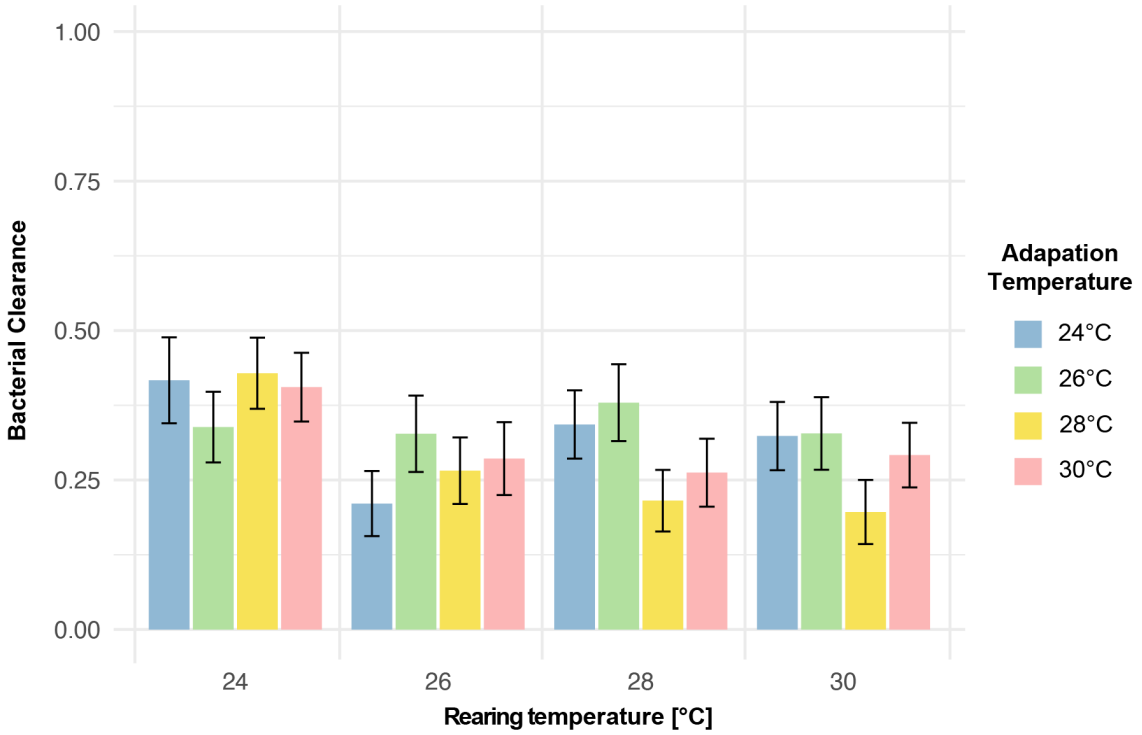

Figure 1: Proportion of individuals that cleared themselves from the bacterial infection 48 hours post inoculation when reared and tested at four temperatures. Each colors represent one adaptation temperature at which mosquitoes were adapted to for 19 generations (Blue: 24°C; Green: 26°C; Yellow: 28°C; Pink: 30°C). Two adaptation lines are pooled together within each adaptation temperature group. Errors bars indication the 95% confidence interval of the mean.

In mosquitoes that did not clear their infection the bacterial load increased with experimental temperature ($\chi^2 = 27.67$, df = 3, $p < 0.001$) (**Figure 2**), with bacterial load being lowest at 24°C and 1.05 to 1.2 higher at 28°C and 30° C than at 24°C and 26 °C (comparisons 24 to 28 and 30 °C: $p < 0.001$); comparisons 26°C to 28°C and 30 °C: $p < 0.06$; comparisons 24°C to 26 °C and 28°C to 30 °C: $p > 0.48$). While the main effect of adaptation temperature was insignificant ($\chi^2 = 0.97$, df = 3, $p = 0.8077$), the interaction between adaptation and experimental temperatures affected bacterial load ($\chi^2 = 18.01$, df = 9, $p = 0.035$). Thus, individuals adapted to 24°C or 30°C harbored about half as many bacteria, if they had been reared at 24°C than if they had been reared at 30°C, and in mosquitoes that had been adapted to 28 °C experimental temperature had no impact on bacterial load.

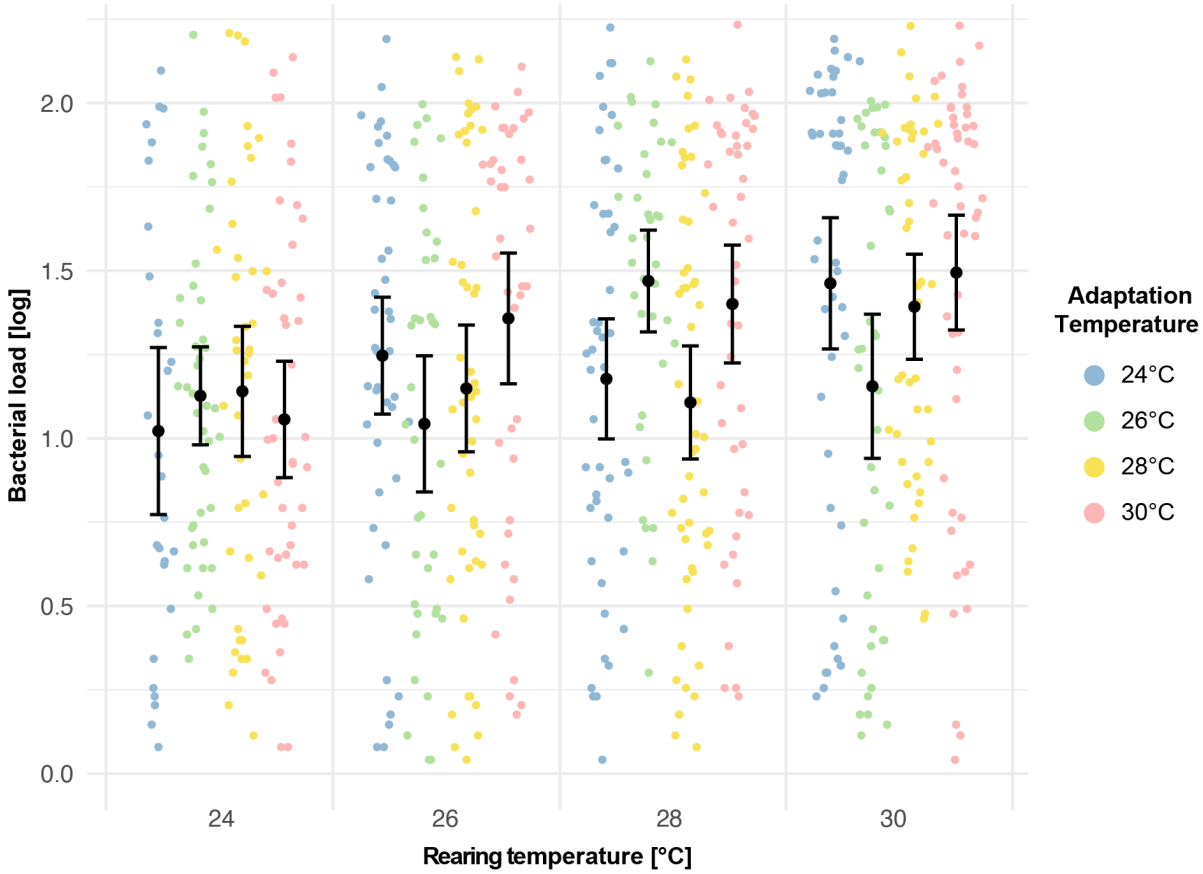

Figure 2: Measured bacterial load (log10) 48 hours after inoculation in function of experimental temperature. Each color corresponds to an adaptation temperature (for 19 generations) - Blue: 24°C; Green: 26°C; Yellow: 28°C; Pink: 30°C. Two adaptation lines are polled together within each adaptation groups. Black dots indicate the mean value for each treatment and error bars the 95% confidence interval around the mean.

Dengue infection

We gave 3920 female mosquitoes the opportunity to fed on a dengue infectious blood meal. Overall, 62.5% ± 1.5% (Mean ± 95% ci) of the females were fully and 78% ± 1.7% survived the 12 days incubation period.

Infection prevalence

Out of the 1305 females that took a successful DENV-1 infectious blood-meal, 87% (95% ci: 85% to 88%) mosquitoes harbored DENV-1 RNA in their body. Overall, infection prevalence was high across all tested temperatures (85 % to 88%) (**Figure 3**) and independent of experimental temperature (χ^2 = 2.115, df = 2, p = 0.347), adaptation temperature (χ^2 = 0.421, df = 2, p = 0.81) and their interaction (χ^2 = 4.576, df = 4, p = 0.334).

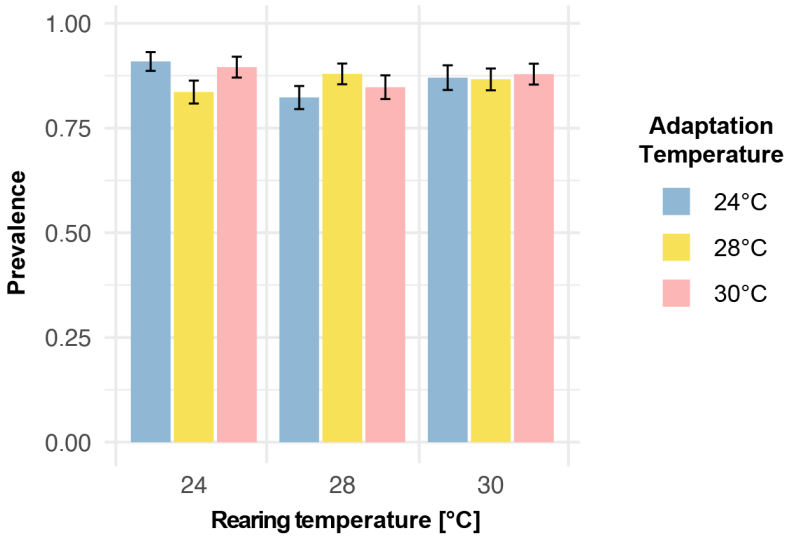

Figure 3: Proportion of individuals harboring DENV-1 in their body 12 days after having taken a DENV-1 infectious blood meal (Infection prevalence). Each color represents one adaptation temperature (Blue: 24°C; Yellow: 28°C; Pink: 30°C). Three adaptation lines are pooled together within one adaptation temperature. Mosquitoes were adapted for 20 generation before being reared and tested at 24°C, 28°C and 30°C. Error bars show the 95% interval confidence of the mean.

Dissemination prevalence

Dissemination prevalence was defined as the number of individuals harboring DENV-1 RNA in their heads over the number of individuals that harbored DENV-1 in their bodies. Dissemination prevalence increased with experimental temperature (χ^2 = 59.85, df = 2, p < 0.001) (**Figure 4a**). Dissemination prevalence was lowest at 24°C (36 % (95% ci: 32% to 41%)) (comparisons 24°C to 28°C and 30°C: p < 0.001), intermediate at 28°C (53% ± 5 %) and highest at 30°C (60% (95% ci: 56% to 65%) (comparison 28°C to 30°C: P = 0.047). Adaptation temperature did not modulate this effect (χ^2 = 2.459, df = 2, p = 0.292) nor was there an interaction between experimental and adaptation temperatures (χ^2 = 7.034, df = 4, p = 0.134).

Disseminated viral load

A similar result was observed for the disseminated viral load measured as the number of viral foci per head (FFU/head). Disseminated viral load increased with experimental temperature (χ^2 = 130.542, df = 2, p < 0.0001) (**Figure 4b**). The disseminated viral load was 1.4 and 1.5 times lower at 24°C than at 28°C and 30°C (comparisons 24°C to 28°C and 30 °C: p < 0.001) and 1.1 times lower at 28°C than at 30°C (comparison 28°C to 30°C: p = 0.0002).The temperature at which mosquito evolved did not influence the intensity of infection (χ^2 = 1.607, df = 2, p = 0.447) nor was there an interaction between experimental and adaptation temperature (χ^2 = 3.208 , df = 4, p = 0.524). In fact, the number of viral foci per head was similar among adaptation temperatures tested at the same experimental temperature.

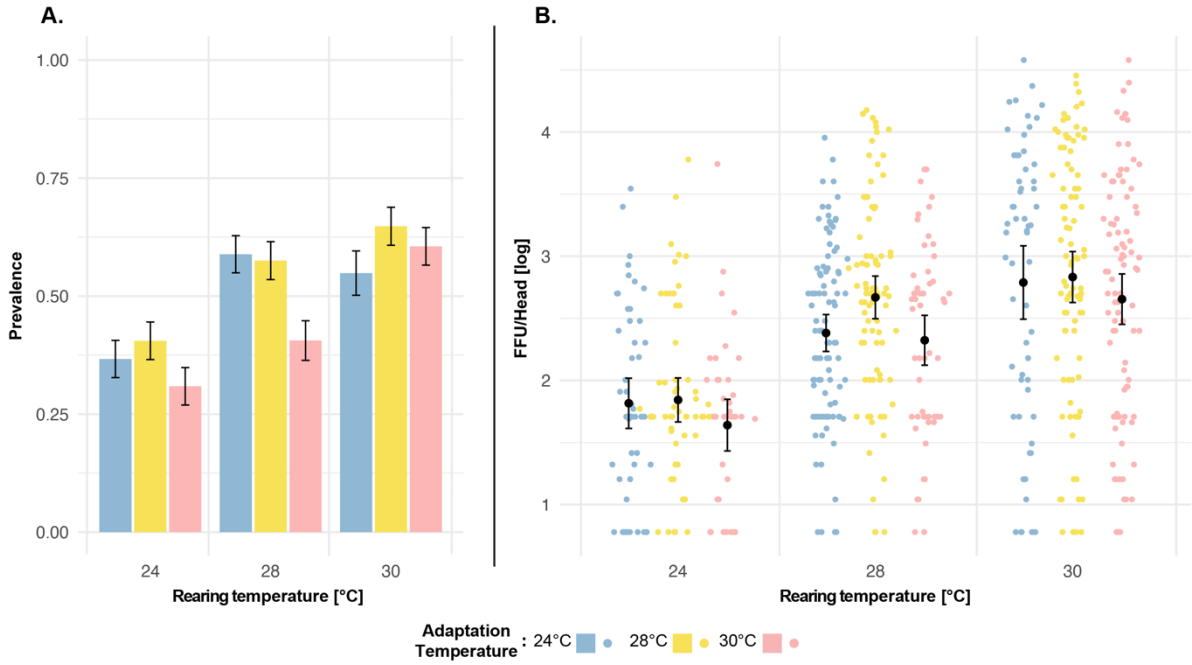

Figure 4: Dissemination of DENV-1 in mosquitoes that were infected with DENV-1 12days prior. Panel A shows the proportion of DENV-1 infected female that harbored virus in their head. Panel B represents the number (log10) of DENV-1 viral foci per head. Black dots represent the mean viral load per treatment. Each color represents one adaptation temperature (Blue: 24°C; Yellow: 28°C; Pink: 30°C). Three adaptation lines are pooled together within one adaptation temperature. Mosquitoes were adapted for 20 generation before being reared and tested at 24°C, 28°C and 30°C. Error bars show the 95% interval confidence of the mean.

Discussion

While the temperature that mosquitoes were reared at influenced the antibacterial response of *Aedes aegypti* and their vector competence for dengue virus, neither changed strongly with the temperature the mosquitoes were adapted to.

We first assessed the antibacterial response with the ability of mosquitoes to clear a bacterial infection and with the bacterial load. Both were strongly influenced by temperature, with greater clearance and lower loads at 24 °C than at 30 °C. Two factors may be responsible for this pattern. First, *E. coli* grows more rapidly at higher temperatures (203,204). While we would therefore expect that bacterial load increases with experimental temperature, it is unlikely that this is the main mechanism underlying our results. Indeed, in LB-medium *E. coli* grows at similar rates between 25°C and 30°C (226), as we have confirmed in a preliminary, unpublished experiment. Second, temperature affects the immune response of insects, including mosquitoes (104,141,202,222,227), for example by changing the expression of immune genes at different temperatures. Thus, our results suggest that the efficacy of the immune response against *E. coli* decreases with increasing temperatures. A possible mechanism is that mosquitoes reared at higher temperatures generally develop more rapidly and emerge earlier (89,169–171) than those reared at lower temperatures, thus decreasing the protein content of teneral

females (95,172) and potentially limiting the resources available for immune functions. The temperature at which mosquitoes were adapted to did not directly influence the anti-bacterial but it did slightly alter the effects of temperature on bacterial load. Whether this is due to direct or indirect effects (e.g., via differences in temperature-dependent developmental time or nutrient uptake) of the adaptation on the immune response is unclear.

We were next interested in testing the potential for thermal adaptation on the ability of females to transmit human pathogens such as the dengue virus. In our setup, infection prevalence was independent of experimental and adaptation temperature which confirms the susceptibility of our mosquito strain for DENV-1 26 26A isolate in laboratory. The lack of variation in infection prevalence suggests that barriers in the midgut were unaffected by our chosen range of temperatures (148). A larger range of temperature is likely to influence this result. Alternatively, it is also possible that our chosen viral load was simply too high to reveal any temperature-dependent patterns.

Dissemination rate and viral load in head tissues increased with experimental temperature regardless of the temperature at which mosquitoes were previously adapted to. Based on prior work, we can expect temperature to modify virus replicate, for example by altering virus structure or affecting genome replication (228–232). Increased viral replication can be expected at higher temperature, it is however unlikely to be the main factor influencing our result. Indeed, in C6/36 mosquito cells, the DENV-1 viral load 10 post-infection is the similar at 24°C, 28°C, and 32°C (233). However, since we here measured DENV-1 infection metrics at a single time-point (12 days post infection), we were unable to estimate the extrinsic incubation period (EIP) of the virus. Based on previous work, we expect the EIP to decrease with increasing temperature, which may also contribute to the observed higher dissemination prevalence and viral load at higher temperature (234).

We've previously argued that differences in anti-bacterial responses with temperature might be related to adult body size and energetical costs of the immune response. A similar argumentative has been postulated before, where adult body was linked to differences in susceptibility of *Aedes aegypti* to viral infection (64). Alternatively, it could also be argued that bigger individuals with higher teneral reserves may provide more intracellular resources for viral replication and production. Although our results do not seem to support this hypothesis it should still be considered for example by measuring DENV-1 infection metrics throughout the mosquito's lifespan.

Finally, as we detected a slight interaction between experimental and adaptation temperature on the ability of mosquitoes to control bacterial growth and because vector competence is largely influenced by the immune response, we expected the same interaction to alter the temperature response of mosquitoes to dengue infection. It is however possible, that density and competition effects stemming

from rearing larvae in groups have dampened the interactive effect between adaptation and experimental temperature.

To summarize, we here provide evidence for differential effects of temperature on the anti-bacterial responses and competence for DENV-1 of the yellow fever mosquito, *Aedes Aegypti* but no to little indication of thermal adaptation between 24°C and 30°C. Understanding how changing temperature will influence vectors physiology is essential if we want to accurately predict future outbreak risks. Based on our results, we conclude that thermal adaptation will not directly alter mosquito vector competence for dengue.

Chapter 5

Unexpected behavioural adaptation of yellow fever mosquitoes in response to high temperatures

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Abstract

Temperature is a major ecological driver of mosquito-borne diseases. Understanding the mosquitoes' thermal biology is essential to inform risk and prediction models of such diseases. Mosquitoes can respond to warming temperatures, for example during climate change, by microhabitat selection through thermal preference. However, it has not yet been considered that mosquitoes are likely to adapt to changing temperatures and alter their preference over evolutionary time. We investigated this by rearing six lines of the yellow fever mosquito *Aedes aegypti* at two temperatures (24°C, 30°C) for 20 generations, and by using these lines to explicitly separate the effects of long-term evolution and within-generation acclimation on their thermal preference in a thermal gradient of 19 - 35 °C. We found that warm-adapted mosquitoes spent 31.5 % less time at high temperatures, which affects their efficiency as vector.

Introduction

With rising global mean temperatures, vector-borne diseases like malaria, dengue and West Nile fever are expected to change in prevalence and geographical range, as both the vectors and the pathogens in the vectors are strongly influenced by temperature (235). To predict how the future climate affects the transmission of vector-borne diseases, epidemiological models are combined with biological data and climate projections (32). While these approaches have provided important conclusions, many ignore that vectors live in a complex world where temperature varies over time and space. In addition, vectors can, at least to some degree, choose the temperature of their habitat, i.e., they have thermal preferences (112,113,236,237), which can potentially evolve.

Fruit flies that were acclimatized for five days to different temperatures did not alter their thermal preference (238), but flies that were reared for 15 generations at warm temperatures preferred warmer locations (239). This sets the stage for unexpected consequences of climate change on vector-borne pathogen transmission due to evolutionary changes of thermal preferences of vectors.

Studies on the effect of climate change on thermal behavior of animals are scarce, and we are not aware of any of such studies on vectors. We hypothesized that mosquitoes selected over several generations at warmer temperatures would more strongly prefer higher temperatures.

Three lines of mosquitoes were adapted to 24°C or 30°C for 20 generations, and the thermal preference of adult females was tested in a thermal gradient of 19 - 35°C as previously described (49,113,236). Thermal preference was defined as the time spent in a temperature zone of the gradient (236). In addition, we determined flight speed and the time flying, because flight allows mosquitoes to select a more suitable temperature. In addition, these parameters allowed us to determine if there was a cold trap, which is the inability to escape due to reduced mobility at low temperatures.

Differences of thermal preference among mosquito lines after long-term adaptation gives an indication of evolution (i.e., genetic changes), whereas differences among rearing temperatures during the last generation gives an indication of acclimation during development (i.e., epigenetic changes).

Materials and Methods

General design

The experiments followed a full factorial design (**Figure 1A**) with six replicates. Laboratory reared *Aedes aegypti* were split into six evolutionary lines: three were maintained for 20 generations at 24 ± 0.3 °C and three at 30 ± 0.3 °C. To assess acclimation, larvae from each line (after the 20 generations

of adaptation) were reared individually at 24 ± 0.5 °C or 30 ± 0.5 °C. For each trial, 10 - 15, females were introduced into the thermal gradient (19 - 35 °C) which was split into five zones. Recorded videos were analyzed using EthoVision video analysis software (236). The behaviors were analyzed using (generalized) linear mixed effect models. The detailed methods are given in the supporting information (SI).

Mosquito maintenance

The mosquitoes used in this study originated from the UGAL strain of *Aedes aegypti* (obtained from Patrick Guérin, University of Neuchâtel, (134)), which has been maintained for many years at 26.5 ± 0.5°C and 75 ± 5 % humidity with a 12:12 hours dark:light cycle. Eggs were hatched at low pressure, and 200 larvae were placed in plastic trays (35 x 21 x 4 cm) filled with 800 ml of deionized water. Larvae were fed with Tetramin Baby® fish food (Tetra GmbH, Melle, Germany) suspended in ddH_2O water (0.06 mg/larva on the day of hatching, 0.08 at day 1, 0.16 at day 2, 0.32 at day 3, 0.64 at day 4, 0.32 per day for older larvae). Pupae were collected and transferred into 300 ml plastic cups containing about 100 ml deionized water, which were placed into a 21 x 21 x 21 cm plexiglass cage. Adults were supplied with 6 % sucrose solution on cotton balls and were given the opportunity to take a blood meal on AK's arm for seven minutes once early (age: 7 days) and once late in life (age: 20 days). 28 hours after the blood meal they were allowed to lay eggs onto filter paper lining the walls of plastic cups (300 ml) filled with 100 ml deionized water. Five days after the blood meal, filter papers were collected, dried, and kept in dark storage at room temperature until being used for the next generation.

Mosquito adaptation

Three lines were adapted at either 24 ± 0.3°C or 30 ± 0.3°C. For each line, the temperature was increased or decreased by 0.7 °C every generation until the target temperature regiment was reached. Humidity and dark:light cycle were not changed. Larvae and adults were kept at the same temperature throughout the adaptation process. Once the target temperature was reached, each line was reared for 20 more generations at the target temperature until they were used in the experiments. In between generations, eggs were stored at room temperature to overcome differences in development time.

Mosquito acclimation

After 20 generations larvae of each line were reared individually in 12-well plates at either 24 ± 0.5°C or 30 ± 0.5°C, both with 75 ± 5% RH and a 12:12 hours dark:light cycle. Each well received 3 ml of deionized water and 0.06 mg Tetramin Baby® suspended in 100 µl water. The feeding schedule was kept the same as in the colony. Pupae of each line and rearing temperature were moved to a Petri

dish with some water, and the Petri dish was placed into a large plastic cup covered with netting. Adults had access to 5 % glucose solution for seven days until the experiment.

Thermal gradient setup and experimental design

Details of the thermal gradient setup are described (113,236). Briefly, it consists of two thermal regulators (AHP-1200CPV, ThermoElectric Cooling America Corporation, Chicago, USA) connected by an aluminum plate (91 × 30 × 2.5 cm, TGB-5030, ThermoElectric) and covered by a custom made, transparent Plexiglas box (80 x 30 x 4 cm). A camera (1/1.8" CMOS sensor, 1280 × 1024, 60fps, acA1300-60gm; Basler, Ahrensburg, Germany) was placed above this setup to film the mosquitoes. The camera was equipped with an infrared-light filter to exclude any changes in lighting in the visible spectrum. Four infrared lights (VAR2-i2-1 short range infra-red illuminator, Raytec, Ashington, UK) were used to light the setup indirectly (minimizing shadow casting). Experiments were performed in a room without windows, but with two LED strips to ensure even lighting. The gradient was chosen to be 5°C above and 5°C below the adaptation temperatures (i.e., 19°C - 35°C). The gradient was applied one hour before experiment started. Temperatures were recorded every five seconds by three dataloggers (MSR145, Seuzach, Switzerland); one at the warm side, one at the cold side and one in the middle of the gradient. The cool and warm sides were switched between experiments to exclude side effects. Salt solutions were used to keep the humidity stable across the gradient (113). The gradient setup was only touched with gloves to prevent contamination with human odors and cleaned each day with 30 % ethanol.

The experimental design followed a full factorial design. One trial consisted of 10 to 15 female mosquitoes of one of the six lines reared at one the two temperatures (24°C and 30°C), yielding 12 trials per block. These trials were repeated six times over six weeks. The order of the trials was randomized within each block. In each trial, the mosquitoes were released in the middle of the gradient and their activity recorded for 15 minutes. The mosquitoes were then removed and killed.

Tracking and video analysis

Videos were analyzed using the EthoVision video analysis software (XT 14, Noldus Information Technology, Wageningen, The Netherlands (240)). This program distinguishes between background and darker individuals to track the movement of individual mosquitoes. This tracking of position over time allows to calculate information such as velocity, distance, and duration of various locomotor behaviors of individuals (241). Video analysis was performed as described previously(236). To get the background image, one of the first 10 frames was used and the recording of movement was only started after all individuals were present in the frame. The arena was subdivided into five temperature zones of 12 x 30 cm (zone 1 - zone 5), with zone 1 always being the coldest zone. Detection settings

were checked for each video but only adjusted when needed, and each tracking record lasted 15 min. The following parameters were analyzed: the cumulative (total) duration spent flying, walking, or resting per individual, the mean distances covered, and the mean velocity. Distinguishing between flying (>1.5 cm/s), walking, and resting (< 0.1 cm/s) was done according to the criteria described by Boyer et al. (241) and Ziegler et al.(236).

Statistical analysis

Trials with fewer than 10 tracked mosquitoes were excluded from data analysis and repeated in an additional block. For the analysis, four datasets were established: one with all data, one with flying data, one with resting data and one with walking data. For each of these datasets, the cumulative duration in each zone [s] and the mean velocity [cm/s] were tested. For model selection, the distribution of each of these variables were checked.

With transformations of log10, mean velocity resembled a normal distribution, which in return allowed for a linear model. Therefore, a linear mixed model was used with adaptation and acclimation temperature as well as temperature zone as explanatory variables. All interactions were included. As random factors, experimental run nested in day, adaptation lines in adaptation temperature and the subject were used. The model with all movement types also included movement type as an explanatory variable.

Since there was a difference in tracking success between groups, cumulative duration in the zones was transformed into relative durations and, therefore, analyzed with a generalized linear mixed model with binomial family and logit link. Explanatory variables and random effects were the same as in the linear mixed model.

For all models, post-hoc-test with multiple pairwise comparison of means with Tukey contrasts were performed on each significant interaction. Analysis of the data was performed in R version 4.1.10 (157) . The lme4 package v. 1.1-31 was used for modeling (224) the ggplot2 package v.3.4.0 for visualization (242), the dplyr package v.2.2.1 for data clean-up (243) .

Results

Adaptation effect

Overall, mosquitoes could be video tracked 91.7 % of the time spent in the arena. Since in all cases adaptation lines did not differ from each other, they were combined (post-hoc, all $P > 0.084$). Mosquitoes adapted to the higher temperature (30 °C) spent 31.5 % less time in the warmest zone than those adapted to 24 °C (generalized linear mixed effect model [GLMM], $chi^2_4 = 57.61$, $P < 0.001$). The same was true when splitting the data into the two acclimation temperatures (24 °C = 36.1 %; 30

°C = 29.5 %; post-hoc, each P < 0.001; **Figure 1B**).The difference between adaptation treatments was largest for flying mosquitoes (19.2 %; $GLMM_{flying}$, chi^2_4 = 29.93, P < 0.001), intermediate for resting mosquitoes (13 %; $GLMM_{resting}$, chi^2_4 = 15.27, P = 0.003) and smallest for walking mosquitoes (6 %; $GLMM_{walking}$, chi^2_4 = 12.90, P = 0.012).

The velocity of flying mosquitoes was influenced by the adaptation temperature (linear mixed effect model [LMM], chi^2_4 = 24.71, P < 0.001), but not the velocity of walking ones (LMM, chi^2_4 = 6.86, P = 0.143). Overall, cold-adapted mosquitoes moved faster than warm-selected ones.

Acclimation effect

The mosquitoes that had acclimated to a higher or to a lower temperature from egg to adults showed a trend that was opposite to the results with the adapted mosquitoes. Mosquitoes acclimated to the warm temperature stayed in the warm zone 14.8 % longer than the ones acclimated to the colder temperatures (GLM, chi^2_4 = 42.10, P < 0.001; **Figure 1B**). The same was true when splitting the data into the two adaptation temperatures (24 °C = 14.6 %; 30 °C = 16.3 %; Post-hoc, each P < 0.001; **Figure 1C**). This was mainly caused by the increase in resting (12.9 %; GLMM, chi^2_4 = 21.86, P < 0.001; Post-hoc, P < 0.001) and walking (10 %; GLMM, chi^2_4 = 20.68, P < 0.001; Post-hoc, P < 0.001) times in the warm zone. No significant effect of acclimation temperature on flying time was found (GLMM, chi^2_4 = 5.03, P = 0.284).

The velocity of walking was 5.8 % higher for mosquitoes acclimated to high temperatures (LMM, chi^2_4 = 24.63, P < 0.001). Cold-adapted mosquitoes were overall faster. No interactions between adaptation and acclimation were found in neither time spent nor velocity.

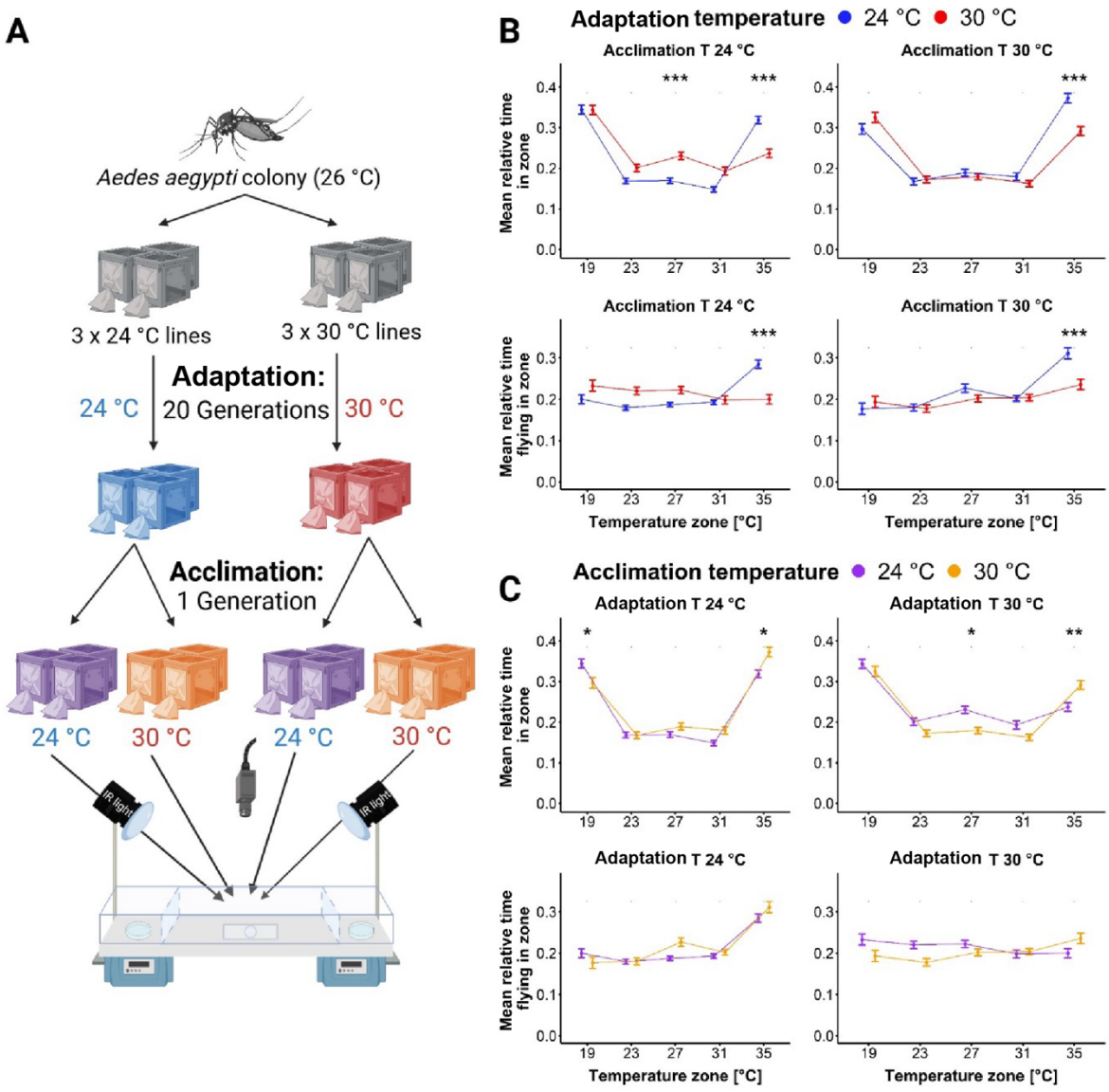

Figure 1: Effect of adaptation and acclimation on thermal preference. A: Scheme of adaptation and acclimation of mosquitoes at different temperatures. B, C: Mean relative time spent by mosquitoes within the temperature zones, for acclimation and adaptation to 24 °C (left) and 30 °C (right), for all movement types (12.4 % flying, 47.5 % resting, 40.2 % walking) (top) or flying (bottom). Data are jittered means over all individuals within a zone with standard errors. Significance (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) is indicated between treatments within one zone (Tukey; 95 % CI). N = 527 reared at 24 °C; 497 at 30 °C; 505 adapted to 24 °C and 519 at 30 °C.

Discussion

Maintaining *Aedes aegypti* for 20 generations at different temperatures affected their thermal preference. In contrast to our hypothesis, rather than preferring the temperature at which the mosquitoes had been adapted to, those adapted to the lower temperature preferred to spend more time in areas with a higher temperature than those adapted to the higher temperature. The effect of

evolution (31.5 %) at high temperatures was about twice as strong as the effect of acclimation (14.8 %).

One mechanism could be that the lines adapted to high temperatures evolved a better heat avoidance response with a higher expression of heat receptors or heat-shock proteins (244–246). Mosquitoes adapted to a high temperature would therefore be better at detecting and avoiding heat, while the low temperature adapted lines lack this capability. In *Aedes aegypti*, the TrpA1 and Ir21a thermal receptors and Ir93a co-receptor play a role in heat detection (245–247). Changes to these receptors are due to epigenetics when the effect is short term, like in acclimation, or due to genetics after long-term adaptation. In our study, both played a role, so that in follow-up studies the relative contributions of epigenetics and genetics underlying expression of these receptors need to be investigated in more detail.

Acclimation temperature also affected the mosquitoes' preference but less than adaptation and in an opposite manner. A similar pattern has been reported for *Culex pipiens*: rearing larvae at a higher temperature increased the critical upper temperature of adults by 1.5 °C (248) .

A higher proportion of individuals was found at both extremities of the setup. Edge effects are expected as mosquitoes generally prefer to rest on corners rather than on open surfaces, and they are difficult to overcome (249). Nevertheless, clear differences between treatments were found.

Our study is the first, to our knowledge, to demonstrate evolutionary changes in the thermal behavior of a vector speciess. Small changes in the temperatures encountered by mosquitoes can have large impacts on their capacity to transmit pathogens (2). Our findings highlight the role of long-term adaptation and short-term acclimation of habitat preferences in response to increasing temperature. A next step is to investigate the genetic changes and the underlying mechanisms of these adaptations, but also to repeat the studies with natural populations and other mosquito speciess.

Since temperature is a major determinant of transmission in models of vector-borne diseases, an inaccurate understanding of how mosquitoes adapt to temperature can under- or overestimate outbreak risks (107). With global temperatures on the rise, it is imperative to gain a deeper understanding of how mosquitoes adapt to their hotter environment by changing their thermal preference and of the mechanisms underlying this adaptation.

Synthesis

Summary of the results

Chapter 1

In this first chapter we were interested in how temperature impacts the immune answer of mosquitoes. For this, we assessed three metrics of the immune answer of mosquitoes that were reared and maintained at four different temperatures – 24°C, 26°C, 28°C and 30°C.

We measured their ability to melanize a negatively charge Sephadex bead, their ability to clear and control a bacterial infection and their survival after an injury. We observed that mosquitoes that were reared and maintained at 28°C and 30°C melanized beads to a lower degree and were less likely to clear a bacterial infection than mosquitoes that were reared and maintained at 26°C and 24°C. Additionally, mosquitoes that were reared and maintained at 30°C were more likely to die in the days following an injury. We conclude that the immune response of mosquitoes is impaired at higher temperatures, possibly due to faster larval development and its consequence on teneral energetic resource reserves.

Chapter 2

In Chapter 2, we focused on testing the interplay between immune responses and the impact of temperature on this interaction. We reared and maintained mosquitoes at four different temperatures – 24°C, 26°C, 28°C and 30°C and assessed their ability to clear and control a bacterial infection (*E. coli*) and to melanize a negatively charged Sephadex in presence or absence of the opposite challenge. We expected the stimulation of one response to impede the other immune response especially at higher temperature. We found that only to be true for the anti-bacterial response. The ability of mosquitoes to control the bacterial infection was lessened when they were challenged with a negatively charged Sephadex bead as opposed to an inert glass bead. In contrast, mosquitoes melanized beads to a higher degree when simultaneously challenge with a bacterial infection. Temperature modulated the phenotype of both response and their interplay. Overall, increasing temperature impede the ability of mosquitoes to mount an effective immune answer, the effect was particularly pronounced for the melanization response.

Chapter 3

In the third chapter of this thesis, we evaluated the thermal response mosquitoes adapted to different temperatures. We let several lines of mosquitoes adapt to either 24°C, 26°C, 28°C and 30°C and tested their thermal response by rearing them at seven different temperatures ranging from 24°C to 34°C. We recorded, their larval mortality, larval developmental time, sex-ratio, longevity, and wing length (as a proxy for body size). We found that the thermal response of all lines largely followed the

previously reported thermal response based on short term exposure to temperature. That is that as temperature increases, developmental time speeds up and body size decreases. However, the temperature at which mosquitoes were previously adapted to, changed the extent of the thermal response and the correlation among traits. The decrease in developmental time and wing size with experimental temperature was stronger in groups adapted to 24°C and 26°C than in groups adapted to 28°C and 30°C. Furthermore, warm adapted mosquitoes also coped better i.e., lived longer at our warmest experimental temperature (34°C) than the cold-adapted ones. Based on those results we conclude that warm-adapted mosquitoes had a higher tolerance to higher temperature and that the effect on longevity is possibly mediated by indirect effects of larval development and body size.

Chapter 4

In chapter four, we investigated the anti-bacterial response and vector competence for dengue of temperature-adapted mosquitoes at different temperature. We found that the temperature at which mosquitoes were reared impacted the anti-bacterial response. The proportion of bacterial clearance was higher in mosquitoes reared at 24°C than in mosquitoes reared at 26°C, 28°C or 30°C. Similarly, bacterial load also increased with rearing temperature, but this effect was stronger in mosquitoes that were adapted to 24°C or 30°C. We next assessed whether the vector competence for dengue of temperature-adapted mosquitoes differed with rearing temperature. The infection prevalence was high (> 85%) regardless of the temperature at which mosquitoes were adapted to and reared at. The dissemination prevalence and the disseminated viral load increased with rearing temperature but weren't impacted by the adaptation temperature. Based on our results, we conclude that thermal adaptation within the thermal tolerance range of mosquitoes might slightly alter the anti-bacterial response but had no effect on the mosquito vector competence for dengue.

Chapter 5

In this last chapter, we tested whether adaptation and acclimation to two temperatures (24°C and 30°C) influenced the thermal preference of mosquitoes. To do so we reared temperature-adapted mosquitoes at those two temperatures and recorded their thermal preference in a thermal gradient ranging between 19°C and 35°C. To our surprise, we found that warm-adapted mosquitoes spend 31.5% less time at high temperature and that overall, they moved slower than cold-adapted ones. In contrast, warm-acclimated mosquitoes- regardless of the adaptation temperature- spent more time (ca. 15%) and moved slightly faster at warmer temperature than cold-acclimated ones. Overall, these results suggest that both short-term and long-term effects of temperature influence thermal preference.

Discussion Chapter 1-2

In the first two chapters of this thesis, we explored the effects of temperature on different immune responses and their interplay in mosquitoes. Together, our result suggest that immune functions become less efficient as temperature increases. We discussed several mechanisms underlying these immune phenotypes. One of which is that temperature influences developmental time and resource accumulation. We argue that higher temperatures hasten larval development and result in smaller less immunocompetent females (201). However, as our results do not substantially differ from previous studies in which larvae were reared at the same temperature (104,106,162,181) , it is likely not the only mechanism involved. To evaluate the relative impact of rearing temperature, adult body size and teneral energetic reserves, further studies may consider rearing mosquitoes at different temperatures and estimating their immune answer at several adult temperatures. We also suggest, estimating the energy budget of teneral females reared under different conditions as well comparing their potential – that is in the absence of an immune challenge- and realized immunity. Indeed, thermal acclimation has been linked to differences in baselevel (potential) immunity and post-challenge immunity (realized) (250). In the second chapter of this thesis, we considered that different branches of the mosquito's immune system can interact with each other and that their interplay can differ with temperature. We specifically tested whether the melanization response and the anti-bacterial response can hinder each other. We observed opposite outcomes for the two responses: the melanization response was enhanced by a simultaneous bacterial challenge and the anti-bacterial response was hindered by a simultaneous melanization challenge. We argued that on the one hand, the melanization response is enhanced because of partially shared immune pathways between the two responses. Indeed, both negatively charged Sephadex beads and bacteria activate the phenol oxidase cascade (162,166,251). On the other hand, bacterial control was potentially limited because of full or partial inhibition of the production of anti-microbial peptides which are essential for the control of persistent bacterial infection (167). We suggest that follow up studies should measure the activation of the three major immune pathways- Toll, IMD and JAK-STAT-, the expression of the different immune effectors and to quantify hemocytes.

Interestingly, although the overall effects of temperature largely followed the same pattern regardless of the challenge, the quantitative details differed. This underscores the sensitivity of our results to environmental conditions.

Discussion Chapter 3-5

In the last three chapters of this thesis, we explored the thermal response of mosquitoes adapted to different temperatures. In chapter three, we assessed how thermal adaptation impacts several life

history traits namely larval mortality, larval development, body size and longevity. The long-term impact (adaptation) of temperature largely followed the extensively described short-term effect of temperature on mosquitoes. In short, as temperature increased, larval development and body size decreased. However, our experiment demonstrated that the quantitative details and correlation among traits differed with adaptation. Overall, our results suggest that warm adapted mosquitoes develop a higher tolerance to high temperature. This was especially apparent for larval development time, body size and longevity. Interestingly, our path analysis indicates that the impact of thermal adaptation on adult longevity is mediated indirectly through its influence on body size. Given that body size is largely dependent on larval conditions (61,252), this suggests that the thermal response of adult mosquitoes is primarily moulded throughout larval development (115). We here focused solely on a uniform thermal environment for both larval stages and adults. However, it is relevant to note that larvae inhabit aquatic environment while adults are in aerial surroundings, leading to distinct ecological niches. Further investigations could explore the effects of heterogeneous larval and adult thermal environment to offer a more comprehensive understanding of thermal adaptation. Finally, while our experiment confirms the potential of mosquito for relatively fast thermal adaptation it would be important to also investigate fecundity of those lines across environmental temperatures as reproductive success is the combination of fecundity and longevity.

As we worked with a vector species, we were also interested in evaluating whether thermal adaptation influences the vector competence of mosquitoes at different temperatures. The results described in chapter 4 suggest that thermal adaptation did not alter the thermal immune response and competence of *Aedes aegypti* for dengue. As ambient temperatures increase, the anti-bacterial response became less efficient and the vector competence for dengue increased. It is however possible that differences among lines might become apparent under more realistic environmental settings. For example, low temperature (24°C) adapted mosquitoes might be cope better with fluctuations at low mean temperature which have previously been shown to accelerate dengue virus transmission (82). Another possibility is for high temperature adapted mosquitoes to cope better with extremely high temperature event (i.e., heat shock). The results of chapter 3 suggest that mosquitoes adapted to 30°C exhibit a higher tolerance for hot temperature. This phenotype might be correlated with the expression of heat shock protein which are to some extent involved in mosquito immune processes (253,254). Moreover, persistence at higher temperatures is also likely to influence the mosquito microbiota. A recent study has shown that the composition of the gut microbiota in *Aedes aegypti* varies with temperature (255). The central role of both larval and adult gut microbiota on mosquito vector competence for arboviruses has been extensively described (reviewed in (256).

For mosquitoes to survive the continuous rise in temperature, they will probably rely on more than evolutionary adaptation alone. It is likely that additional measures such as behavioural thermoregulation or short-term plastic responses based on heat shock protein increase the range of thermal tolerance and reduces the extent of evolutionary thermal adaptation (49,50,113,114,244,257,258). In chapter 5, we considered the possibility that thermal preference can also change with thermal adaptation. Our results are surprising and suggest that warm-adapted mosquitoes avoided warm temperature more than cold-adapted ones. On the other hand, acclimation showed an opposite pattern although the difference was roughly half as pronounced as the difference related to adaptation. We recommend further studies to consider the influences of both acclimation and adaptation on heat detecting receptors such as the TrpA1 and Ir21a thermal receptors, and Ir93a co-receptor (245–247).

The implications of our results on vectorial capacity and disease outbreak risk are difficult to predict. On the one hand, adaptation to warmer temperature led to a higher tolerance for high temperatures. As longevity is a main determinant of vectorial capacity (53) , higher survival at high temperature will augment vectorial capacity. We found no evidence of thermal adaptation for immune functions and vector competence for dengue, but both were strongly affected by environmental temperatures. Immune functions were impaired at higher temperature and dissemination prevalence and disseminated viral load both increased with environmental temperature, probably leading to more transmission. On the other, the preference for colder temperatures of warm-adapted mosquitoes might mitigated the positive effects of longevity and vector competence on vectorial capacity. Our work highlights that considering only the short-term responses of mosquitoes to temperature can lead to both over and underestimation – depending on the specific situation- of disease risk in a changing climate.

Conclusion

This thesis has investigated the effects of temperature on a wide range of behavioral, physiological and fitness traits of the yellow fever mosquito *Aedes aegypti*. Altogether, our results contribute to a broader understanding of the short-term and long-term thermal response of mosquitoes. The consequences of thermal acclimation and adaptation on vectorial capacity remain unclear and need further consideration and underlie the necessity for a better consideration of thermal adaptation in the epidemiology of vector-borne diseases in the context of climate warming.

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