Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2025:45
Faculty of Landscape Architecture, Horticulture
and Crop Production Science

Bridging environmental stress and internal physiology to mosquito biology

Life-history, molecular biology and neuroethology

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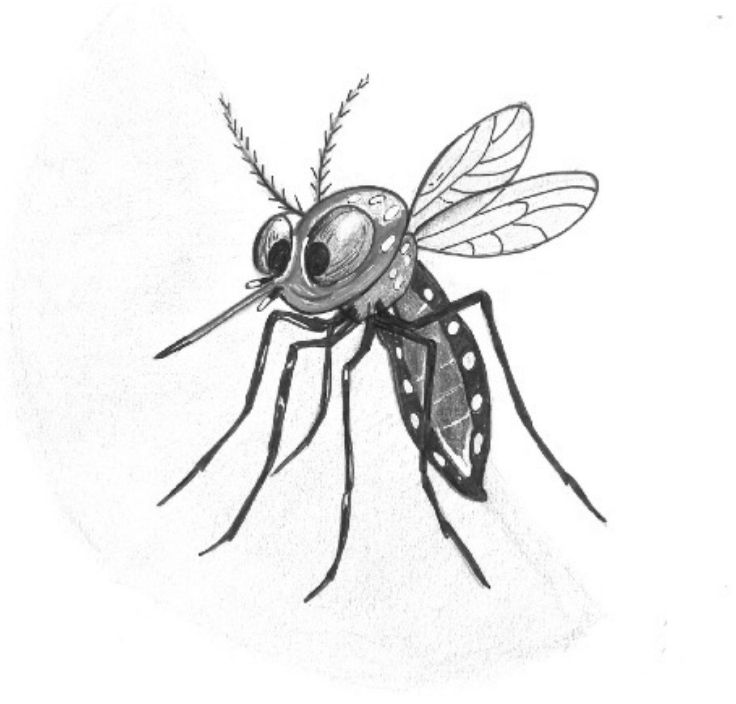

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SLU
SWEDISH UNIVERSITY
OF AGRICULTURAL
SCIENCES

DOCTORAL THESIS

Alnarp 2025

Acta Universitatis Agriculturae Sueciae
2025:45

Cover: Mosquito pencil drawing by Sukritha

ISSN 1652-6880
ISBN (print version) 978-91-8046-480-2
ISBN (electronic version) 978-91-8046-530-4
https://doi.org/10.54612/a.29djgcqihk

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Print: SLU Grafisk service, Alnarp 2025

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Abstract

Mosquitoes rely on diverse internal and external cues to regulate key physiological processes and behaviour. Climate change alters the distribution and behaviour of mosquitoes, including *Aedes aegypti*, a primary vector of dengue and yellow fever. However, how vectoring females respond to climate change drivers, such as elevation in CO_2, and abiotic stressors, including desiccation, remains unclear. This study shows that exposure to elevated CO_2 levels combined with extended egg desiccation periods differentially alters larval development and survival, with carry-over effects on adult life-history traits and feeding behaviour (Paper I). Female mosquitoes use their peripheral olfactory system to locate nectar and blood resources. Transcriptomic and functional ontology analyses of olfactory tissues, the antennae and maxillary palp, reveal significant changes in gene expression related to stress and chemosensation, particularly in the CO_2-sensing maxillary palp, in response to elevated CO_2 and extended egg quiescence (Paper II). Collectively, these findings show that climate change can impact mosquito population dynamics and adult foraging behaviours. Teneral females are attracted to floral sources to replenish their energy reserves, while ageing induces a gradual shift to host-seeking, correlated with a concerted increase in chemosensory receptor expression, with exceptions, such as odorant receptor, *Or117* which follows an inverse expression pattern. The mechanism underlying *Or117* gene expression and age-dependent floral-seeking was investigated through behavioural assays, electrophysiology, receptor characterisation and functional genomics. Females display an age- and mating-state-dependent floral seeking behaviour, in which Or117 and its ligand, camphor are required for the attraction in teneral unmated females (Paper III). As a whole, the findings broaden our understanding of mosquito adaptation to internal cues and environmental stressors, with implications for vector ecology and disease transmission.

Keywords: Carbon dioxide, Egg quiescence, Climate change, *Aedes aegypti,* Life-history, Olfactory system, Transcriptome, Floral-seeking, Internal state, CRISPR-Cas9

Integration av miljömässig stress och intern fysiologi i förståelsen av myggans biologi

Abstrakt

Myggor svarar på en rad interna signaler och yttre miljömässiga faktorer som reglerar viktiga fysiologiska processer och beteenden. Klimatförändringar påverkar myggors beteende och utbredning, inklusive *Aedes aegypti*, en huvudvektor för dengue och gula febern. Det är dock fortfarande oklart hur honor av denna art reagerar på klimatrelaterade faktorer, som ökade koldioxidnivåer (CO_2) och abiotiska stressfaktorer som uttorkning. Denna studie visar att exponering till förhöjda CO_2-nivåer i kombination med förlängda perioder av uttorkning av äggen påverkar larvutveckling och -överlevnad på olika sätt. Detta har även kvarstående effekter på vuxna individers livshistorieegenskaper och födointag (Paper I). Honmyggor använder sitt perifera luktsinne för att lokalisera nektar- och blodkällor. En transkriptomanalyser av myggans luktorgan – antenner och maxillarpalper – visar på betydande förändringar i genuttryck relaterat till stress och detektion av kemiska signaler, särskilt i den CO_2-känsliga maxillarpalpen (Paper II), som svar på höga CO_2-nivåer och förlängd äggdvala. Tillsammans visar dessa resultat på att klimatförändringar kan signifikant påverka myggpopulationers dynamik och vuxna individers födobeteenden. Nykläckta (tenerala) honor attraheras främst av blommor för att återställa sina energireserver, medan äldre honor gradvis övergår till att söka värddjur – en förändring som är kopplad till ett samordnat ökat uttryck av kemoreceptorer. Vissa receptorer, som doftreceptorn Or117, uppvisar dock ett omvänt uttrycksmönster. Mekanismen bakom Or117:s genuttryck och den åldersberoende blomattraktionen undersöktes med hjälp av beteendeanalys, elektrofysiologi och funktionell genomik. Honmyggors attraktion till blomdoft var ålders- och parningsberoende, där Or117 och dess ligand krävs för attraktion hos tenerala, oparade honor (Paper III). Sammantaget tillåter dessa resultat en fördjupad förståelse för hur myggor anpassar sig till både interna signaler och miljöstressfaktorer, vilket kan påverka såväl vektorernas ekologi som sjukdomsspridning.

Keywords: Koldioxid, Äggdvala, Klimatförändringar, *Aedes aegypti*, Livshistoria, Luktorgan, Transkriptom, Blomsökande, Inre tillstånd, CRISPR-Cas9

Dedication

To Amma, Acha, Abhi — and to all the women who walked before me, with me, and those yet to come.

> “You teach me, I forget. You show me, I remember. You involve me, I understand.”
> — Edward O. Wilson

> “Somewhere, something incredible is waiting to be known.”
> — Carl Sagan

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Sukritha Nalikkaramal**, Sharon Rose Hill & Rickard Ignell (2025). Effect of extended egg quiescence and elevation in carbon dioxide on life history traits of *Aedes aegypti*. Scientific Reports, 15 (9310), https://doi.org/10.1038/s41598-025-92193-4

II. **Sukritha Nalikkaramal**, Sharon Rose Hill & Rickard Ignell (2025). Impact of elevated CO_2 and extended egg quiescence duration on gene expression in the peripheral olfactory system of *Aedes aegypti*. Scientific Reports, 15 (14318), https://doi.org/10.1038/s41598-025-98159-w

III. **Sukritha Nalikkaramal***, Julien Pelletier*, Anaïs Karine Tallon, Betelehem Wondwosen, Lukas Weiss, Sharon Rose Hill & Rickard Ignell. Odorant receptor Or117 regulate floral seeking in female *Aedes aegypti* (manuscript)

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* indicate equal contribution

The contribution of Sukritha Nalikkaramal to the papers included in this thesis was as follows:

I. Designed the methodology along with the co-authors. Performed all data collection and data analysis. Drafted the manuscript with inputs from all co-authors.

II. Conceptualised the idea with the co-author. Designed the methodology along with the co-authors. Performed all data collection and data analysis. Drafted the manuscript with inputs from all co-authors.

III. Designed the methodology on mosquito behaviour with the co-authors. Performed all data collection and data analysis on behavioural assays. Drafted the final manuscript with co-authors.

List of figures

Abbreviations

AL	Antennal lobe
Cas9	CRISPR-associated protein 9
CO_2	Carbon dioxide
CRISPR	Clustered regularly interspaced short palindromic repeats
CSP	Chemosensory protein
DEG	Differentially expressed genes
Gr	Gustatory receptor
Ir	Ionotropic receptor
LH	Lateral horn
LN	Local interneuron
MB	Mushroom body
OBP	Odorant binding protein
Or	Odorant receptor
Orco	Odorant receptor co-receptor
OSN	Olfactory sensory neuron
PN	Projection neuron
PPK	Pickpocket receptor
SNMP	Sensory neuron membrane protein
TRP	Transient receptor potential
VOC	Volatile organic compound
WHO	World Health Organisation
ZT	Zeitgeber time

1. Introduction

Mosquitoes (Diptera: Culicidae) are recognised as the deadliest animals to humans due to the pathogens transmitted by blood-feeding females. Of the more than 3,700 mosquito species identified worldwide, only approximately 2.5% are competent vectors of human disease-causing pathogens (Yee *et al.,* 2022). Despite the small proportion of disease-vectoring species, this subset, including species, such as *Aedes aegypti* and *Anopheles gambiae,* is a significant public threat. Collectively, these species place an estimated 80% of the global population at risk of one or more vector-borne diseases (WHO, 2017; WHO, 2024). Among these diseases, malaria, transmitted by *Anopheles* mosquitoes, remains a major global concern, with an estimated 263 million cases reported in 2023 (WHO, 2024). Concurrently, the incidence of dengue, predominantly transmitted by *Ae. aegypti*, has surged in recent years, contributing to the growing global socio-economic burden (Bhatt *et al.,* 2013; WHO, 2024). In addition to dengue, *Ae. aegypti* is also a primary vector of other viral diseases, including yellow fever, Zika and chikungunya, all of which show increasing incidence and expanding geographical ranges (Charrel *et al.*, 2014; Weaver, 2014; Kraemer *et al.*, 2015). The transmission dynamics of these viral pathogens are closely tied to the population dynamics of *Ae. aegypti*, which in turn depends on the plastic behaviour and adaptation to climatic factors of this species (Kraemer *et al.*, 2015; Messina *et al.*, 2019).

The anthropophilic nature of *Ae. aegypti* has been shaped by a suite of key evolutionary pressures (Brown *et al.*, 2014; Rose *et al.*, 2020; Rose *et al.*, 2023). A critical driver of the domestication of *Ae. aegypti* was the species' transition to utilising human-derived water sources for oviposition and larval breeding, particularly during periods of climatic desiccation in sub-Saharan Africa (Rose *et al.*, 2020). This ecological shift was accompanied by the evolution of other adaptive traits, such as selective egg hatching in response to environmental cues typical of human-altered habitats and exceptional desiccation tolerance, features that have underpinned its successful geographic expansion and urban specialisation (Diniz *et al.*, 2017; Rose *et al.*, 2020; Hillery *et al.*, 2022). In addition to external environmental adaptations, female *Ae. aegypti,* as well as other species studied, exhibit remarkable behavioural plasticity in response to internal physiological states (Hill and Ignell, 2021). Such phenotypic plasticity likely plays a crucial role in facilitating both short-term survival and long-term evolutionary success across diverse ecological contexts (Agrawal, 2001).

The vectorial capacity of mosquitoes is inherently tied to the feeding behaviour of females and the ensuing population dynamics (Brady *et al.*, 2015; Kramer and Ciota, 2015). Despite substantial progress in understanding mosquito biology, gaps remain in elucidating how environmental and internal factors modulate the physiology and behaviour of disease-vectoring mosquitoes. This thesis addresses these knowledge gaps by investigating how external environmental stressors, such as elevated atmospheric CO_2 and extended periods of desiccation, as well as female internal physiological state, including aging and mating status, modulate the physiology and behaviour of *Ae. aegypti*. The studies presented herein integrate ecological, functional genomic and neuroethological approaches to dissect the mechanistic underpinnings of these effects. Collectively, these findings advance our understanding of the environmental and physiological drivers of mosquito behaviour and physiology, with direct implications for vector biology and disease transmission dynamics.

2. Background

2.1 From eggs to adult hunters

Mosquitoes are holometabolous insects, whose immatures stages, including larvae and pupae, are aquatic, while adults are terrestrial. Species from the Aedini tribe, including *Ae. aegypti,* lay their eggs near ephemeral water bodies, such as potholes and discarded tyres (Powell and Tabachnick, 2013). Within a gravid mosquito, egg development involves the sequential secretion of two protective layers, the endochorion and the exochorion, by the maternal follicular cells (Mathew and Rai, 1974; Clements, 1999). Upon oviposition, *Ae. aegypti* eggs are initially prone to water loss. However, the eggs rapidly develop desiccation resistance through processes involving sclerotisation, in which chorionic proteins cross-link and harden, as well as undergo melanisation (Clements, 1999). Additionally, during embryogenesis, extra-embryonic cells produce a serosal cuticle inside the chorion, further enhancing the desiccation tolerance (Clements, 1999; Li and Li, 2006; Rezende, *et al*., 2008). Embryogenesis is typically completed within 2-to-4 days post-oviposition, depending on ambient temperature (Farnesi *et al*., 2009). This is followed by a phase of reduced metabolic activity in which fully-developed pharate larvae remain within the egg in a desiccation-resistant state, a phenomenon known as egg quiescence (see 2.2.1. Desiccation and dormancy).

While hatching from quiescence is primarily triggered by rehydration, additional signals, including decreasing oxygen levels, microbial activity and optimal temperature, are required to initiate successful emergence (Gjullin *et al*., 1941; Clements, 1999). Newly hatched pharate larvae represent one of the most vulnerable stages of the lifecycle of a mosquito, as they must quickly adjust to environmental changes when they transition from relying on internal reserves to actively feeding on available resources. Breeding sites are often unstable, subject to fluctuations in water volume, nutrient content, pH, presence of toxic metals, predators and competition (Clements, 1999; Clark *et al*., 2004; Perez and Noriega, 2012; Talaga *et al*., 2020; Kumar *et al*., 2024). Such environmental stressors can profoundly influence larval development and survival (Perez and Noriega, 2012; Kumar *et al*., 2024).

During the larval stage, *Aedes* larvae feed on organic materials and microorganisms (Merritt *et al.*, 1992), accumulating a threshold energy reserve and metamorphose into a non-feeding pupal stage (Chambers and Klowden, 1990).

The larval environment can lead to carry-over effects on emerging adult survival, body size and energy reserves, which can influence female resource-seeking decisions critical for the disease transmission dynamics (Moller-Jacobs *et al.*, 2014; Chandrasegaran *et al.*, 2020; Paper I). The developmental trajectory of *Ae. aegypti* from egg to adult is thus profoundly shaped by early-life environmental conditions, particularly during egg quiescence and larval development (Vinauger and Chandrasegaran, 2024). Understanding how environmental variability during developmental time translates into phenotypic plasticity is thus essential in predicting mosquito population dynamics and disease transmission potential.

2.2 Influence of environmental factors

Under changing climatic conditions, much focus has been given to the effects of the increase in temperature on mosquito physiology; however, several other factors, such as elevation in CO_2, changing rainfall patterns or water availability, also have the potential to influence mosquito biology. The success of mosquito species, such as *Ae. aegypti*, has been attributed to rapid adaptation to environmental changes through plastic life history traits, with serious implications for vector competence. This section will briefly address the two environmental factors: egg quiescence, in the context of water availability, and elevation in CO_2 levels.

2.2.1 Desiccation and dormancy

Insects are prone to desiccation due to their high surface area to volume ratio (Hadley, 1994). Thus, adaptation to changing atmospheric humidity or water availability through desiccation tolerance is crucial (Holmes and Benoit, 2019). While desiccation-tolerant dormancy periods can occur at different life stages of a mosquito, including egg, larva or adult, in *Ae. aegypti* this is predominantly observed in the egg stage (Diniz *et al.*, 2017). Notably, desiccation-tolerant dormancy can be of two types: quiescence, a non-seasonal, non-programmed state of reduced metabolism, and diapause, a seasonal programmed state, both of which are adaptations to unfavourable environmental conditions (Diniz *et al*., 2017). In line with the scope of this thesis, this section will focus on egg quiescence in *Ae. aegypti*.

Across evolutionary timescales, eggs have developed several adaptations to withstand periods of sub-optimal conditions. These include the formation of the serosal cuticle (Rezende *et al*., 2008), increased chitin formation (Clements, 1999) and altered metabolism (Prasad *et al*., 2023). While egg desiccation duration is dependent on various environmental factors, such as temperature and humidity (Farnesi *et al*., 2009), egg survival depends on morphological adaptation in the egg, as well as inherent maternal energy reserves (Diniz *et al*., 2017, also see 2.3.1. Energy reserves across life stages).

Niche container-breeders, such as *Aedes*, are often exposed to abrupt and drastic fluctuations in water availability and other microclimatic conditions (Clements, 1999; Diniz *et al*., 2017). The desiccation-tolerant eggs of *Ae. aegypti* can survive harsh environments up to 3 months, without affecting hatching rates (Oliva *et al*., 2018). Population-level variation in egg quiescence period within *Ae. aegypti*, indicate differential adaptations that are intrinsically regulated under different environmental pressures (Trpis, 1972; Oliva *et al*., 2018). The differential success of *Ae. aegypti* over the invasive *Aedes albopictus*, at breeding habitats, has been linked to increased tolerance to desiccation, bypassing dry seasons (Juliano *et al*., 2002). Overall, extended quiescent periods pose an evolutionary advantage for *Ae. aegypti* in the microhabitats and at the global scale for range expansion (Diniz *et al.,* 2018; Oliva *et al*., 2018).

While quiescent periods may be advantageous (Yang, 2014), prolonged periods can reduce the maternal energy reserves, causing an imbalance in metabolic costs in pharate larvae (Perez and Noriega, 2012). Extended egg quiescent periods can increase the susceptibility of the emerging larvae to abiotic stressors, such as elevation in CO_2 (Paper I), metal toxins (Perez and Noriega, 2012), as well as alter adult survival (Perez and Noriega, 2013) and insecticide resistance (Sachez, 2021). In insect species displaying early life stage-dormancy, the life history phenotype of emerging larvae displays anticipatory adaptive changes to accommodate changes in the environment, called anticipatory phenotypic plasticity (Esperk *et al.,* 2012; Perez and Noriega, 2013). For an *Ae. aegypti* egg under quiescence, any prolongation of quiescence could be a cue of the sub-optimal conditions that the pharate larvae have to inhabit. While less explored, anticipatory phenotypic plastic response to adapt to abiotic stressors, such as metal toxicity, has been observed in life history traits of *Ae. aegypti* adult emerging from extended egg quiescence (Perez and Noriega, 2013). Comparable effects have also been observed in adults, with increased survival under elevated CO_2 levels when emerging from eggs subjected to prolonged quiescence (Paper I). However, the underlying mechanism of such adaptive response remains unclear.

2.2.2 Anthropogenic increase in CO_2 levels

Atmospheric CO_2 levels have been steadily rising and are projected to increase exponentially, reaching approximately 1000 ppm by the end of the century, up from the current ambient level of ~420 ppm (IPCC, 2018). For aquatic ecosystems, including freshwater bodies, this escalation translates into an increased partial pressure of CO_2, leading to acidification and alterations in water chemistry (Hasler *et al.*, 2017; Weiss *et al.*, 2018). These physicochemical changes can cause physiological stress, including hypercapnia and oxidative stress, on freshwater organisms, affecting critical life history traits, such as survival, development, reproduction, as well as sensory function (Michaelidis *et al.*, 2005; Kurihara, 2008; Munday *et al.*, 2009; Munday *et al.*, 2010; Fitzer *et al.*, 2012; Abbey-Lambertz *et al.*, 2014; Chung *et al.*, 2014; Kowalewska *et al.*, 2020; see also 2.6.2. The role of external environment).

In stagnant water bodies, such as those exploited by mosquito larvae, the effects of elevated CO_2 and associated acidification may be pronounced, impacting larval physiology and survival (Tuchman *et al.*, 2003; Paper I). Direct effects of elevated CO_2 exposure have been shown to reduce larval survival, delay development and affect adult life history traits in mosquitoes (Paper I). Indirect consequences of elevated CO_2 on mosquito larvae have also been studied through their effects on plant litter quality. For instance, Tuchman *et al.* (2003) demonstrated that larvae feeding on leaf litter grown under elevated CO_2 conditions exhibited increased mortality and prolonged development duration. In contrast, other studies reported no significant effects on larval performance (Strand *et al.*, 1999; Alto *et al.*, 2005). These findings highlight the importance of disentangling direct physiological effects from indirect ecological effects to better predict mosquito physiology for predicted climate changes.

2.3 Energy reserves and metabolism

Across all living organisms, nutrient acquisition, synthesis of necessary reserves and efficient utilisation determine individual success and population dynamics. Insects constantly require energy during foraging activities, as well as for general homeostasis (Arresse and Soulages, 2010). For holometabolous insects, the availability and quality of nutrients during early life stages profoundly shape life-history traits, such as developmental duration, survival, adult size, reproductive fitness and feeding behaviour (Nestel *et al.,* 2016; Teder and Kaasik, 2023). In mosquitoes specifically, maternal nutrient investment into eggs significantly influences the overall fitness of the emerging immature and adult stages (Zirbel and Alto, 2018; Yanchul and Alto, 2021). These energy reserves serve as a foundation bridging environmental resource availability and mosquito biological outcomes.

2.3.1 Energy reserves across life stages

Embryogenesis is an energy-intensive process, requiring maternal metabolic reserves to fuel cell division and differentiation, as eggs function as a closed energy system (Nestel *et al.,* 2016). In the desiccation-tolerant quiescent eggs of *Ae. aegypti*, lipid reserves decline as a function of quiescent duration (Perez and Noriega, 2012; Prasad *et al*., 2023). Additionally, the pharate first instar larvae inside the eggs enter a hypometabolic state, characterised by metabolic rewiring, ensuring survival of the pharate larvae, both during desiccation and subsequent hatching (Diniz *et al*., 2017; Prasad *et al*., 2023). This metabolic adaptation is a conserved mechanism among other dormant *Aedes* life stages, including temperate diapausing populations of *Ae. aegypti*, wherein dormant larvae exhibit reduced energy metabolism and increased lipid accumulation (Reynolds *et al*., 2012; Batz and Armbruster, 2018; Mensch *et al*., 2021). In diapausing or quiescent eggs, there is an upregulation of genes involved in long-chain fatty acid and polyamines biosynthesis (Urbanski *et al*., 2010; Reynolds *et al*., 2012; Prasad *et al*., 2023), which aid in desiccation tolerance (Benoit and Denlinger, 2007;

Juarez and Fernandez, 2007) and likely ensure survival of emerging larvae on rehydration.

Following hatching, larval nutrient acquisition is crucial for both immediate energy demands and reserve synthesis, primarily in the form of glycogen and lipids (Arresse and Soulages, 2010; Telang *et al.*, 2007). The insect fat body, functionally analogous to the vertebrate liver, plays a central role in nutrient sensing, metabolic regulation and energy storage (Arresse and Soulages, 2010). Different mosquito species exhibit distinct metabolic strategies during larval development to adapt to the ecological niche they occupy (Timmermann and Briegel, 1999). *Ae. aegypti* larvae prioritise lipogenesis, as evident from exponential fat accumulation, whereas glycogen and protein synthesis follow a more linear trajectory with larval growth (Timmermann and Briegel, 1999). Notably, ~50% of the larval diet is utilised for lipogenesis and ~35% for glycogenesis (Zhou *et al.*, 2004). This strategy has likely evolved to ensure maximum energy storage during a developmental "window of opportunity" to ensure survival during food shortages (Zhou *et al.*, 2004). Furthermore, this ensures critical energy reserves necessary for metamorphosis into a non-feeding pupal stage that relies entirely on stored reserves (Chambers and Klowden, 1990; Telang *et al.,* 2007). The duration of larval feeding, particularly during late instars, directly impacts pupation commitment and energy reserves carried over from pupae to adult stages (Telang *et al.*, 2007).

Across the development of holometabolous insects, including mosquitoes, the metabolic trajectory, which is assessed by measured CO_2 emission rates post-puparium formation, is characterised by high metabolic rates at the onset of metamorphosis, a pronounced decline during mid-metamorphosis and a final increase before adult emergence (Merkey *et al.,* 2011). In *Drosophila melanogaster*, a change in environmental temperature did not disrupt the overall metabolic pattern during pupae-to-adult transition but caused increased energy expenditure at temperature extremes (Merkey *et al.,* 2011). Such metabolic shifts during this critical developmental time window can interfere with processes essential for successful adult emergence, including the development of the sensory system (see 2.6.1. Regulation of chemosensory gene expression). Teneral adult mosquitoes retain a proportion of the larval-derived energy reserves, which sustain early post-emergence activities, such as foraging flight (Clements, 1955) and even contribute to first egg batch production (Telang and Wells, 2004). Environmental conditions during larval development, both abiotic, *e.g*., temperature, pH and CO_2 concentration, and biotic, *e.g*., population density and nutrient availability, can have carry-over effects on adult traits, such as adult survival, body size and metabolic reserves (Takken *et al*., 1998; Briegel *et al*., 2001; Clark *et al*., 2004; Moller-Jacobs *et al*., 2014; Huxley *et al*.,2021; Huxley *et al*., 2022; Paper I). Thus, under optimal growth conditions, adult body size, which strongly reflects larval nutritional status, can serve as a robust proxy for assessing larval environmental quality, with direct implications for adult metabolic reserves and resource-seeking behaviour. (Briegel 1990; Briegel *et al*., 2001).

Accumulated teneral adult glycogen and lipid reserves primarily serve as energy sources (Zhou *et al.,* 2004). Fat body transcriptomic and metabolomic studies reveal that mosquitoes with reduced body size have altered expression of genes involved in immunity, reproduction and metabolism (Price *et al*., 2015). Notably, smaller females display reduced yolk protein synthesis following a blood meal, suggesting a trade-off between nutrient utilisation and reproductive benefit (Price *et al.,* 2015). These findings, in the context of other life history studies, suggest that smaller females compensate for sub-optimal stored reserves through differential utilisation of feeding resources and metabolism rate (Takken *et al.,* 1998; Takken *et al.,* 2013). Reserve replenishment in adults can occur through either sugar or blood feeding (Van Handel and Lea, 1970; Van Handel, 1965). In *Ae. aegypti* females, independent of body size, early-life access to only blood has been shown to enhance the energy reserves to a higher level compared to females fed on both blood and sugar (Naksathit *et al.,* 1998b). While early-life access to sugar and/or blood can initiate reserve synthesis, which generally enhances survival (Nayar & Sauermann, 1975), the allocation of imbibed resources to longevity and reproductive benefits might have species-specific patterns (Nayar & Sauermann, 1975c; Gary and Foster, 2001; Van Handel and Lea, 1970; Zhou *et al*., 2004).

Older starved *Aedes* females exhibit increased lipogenesis from an equal calorie meal of sugar or blood (Van Handel, 1965a; Briegel, 1990), though glycogenesis proceeds more rapidly in sugar-fed females (Van Handel and Lea, 1970). A starved *Aedes* female can imbibe up to two-thirds of her weight in sugar meal, resulting in a substantial increase in lipid and glycogen levels

(Van Handel *et al.,* 1965). Fundamentally, a teneral *Aedes* adult possesses higher energy reserve content compared to *Anopheles*, which has profound consequences on adult resource allocation. A sugar meal is efficiently utilised by both *Aedes* and *Anopheles* females. However, unlike *Aedes*, *Anopheles* display less efficient blood meal utilisation for oogenesis, as a substantial portion of blood protein is utilised for maternal reserve synthesis (Briegel, 1990). In *Aedes*, energy reserve synthesis can be replenished either through a single, high-concentration sugar meal (Nayar and Sauerman *et al.,* 1975) or via frequent feeding over time (Naksathit *et al.,* 1999a). When compared to *Aedes*, *Anopheles* females emerge with limited teneral reserves and often use the first blood meal for maternal reserve synthesis rather than immediate egg production. This results in multiple blood-feeding cycles as a reproductive strategy (Briegel 1990b; Briegel and Hörler, 1993), a pattern also observed in nutrient-deprived *Ae. aegypti* females, who may supplement with sugar feeding or increase blood feeding frequency (Feinsod and Spielman *et al.,* 1980). Conversely, *Anopheles* females with ample energy reserves can support oogenesis with smaller blood meals (Briegel, 1990a). This dynamic relationship between teneral reserves, feeding frequency, and resource utilisation underlies the vectorial efficiency of *Ae. aegypti* and *An. gambiae*. (Stone and Foster, 2013).

The size and nutritional status of female *Ae. aegypti* significantly influence maternal reserve contributions to the eggs, a factor critical for the survival of desiccation-resistant quiescent eggs (Briegel, 1990a; Diniz *et al*., 2017). During the first gonotrophic cycle, teneral reserves, as well as sugar and blood feeding, collectively contribute to protein and lipid accumulation in the egg (Briegel *et al*., 2001; Zhou *et al*., 2004). Prolonged dormancy or quiescence can be stressful for the pharate larvae as a result of limited energy reserves, oxidative stress and increased reactive oxygen species (Diniz *et al*., 2017; Cornette *et al*., 2023). As a result, females emerging from extended egg quiescence had reduced reproductive output (Perez and Noriega, 2013). Moreover, the larvae offspring hatched from eggs laid by females that experienced prolonged egg quiescence exhibited reduced starvation tolerance, likely via a maternal effect (Perez and Noriega, 2013). The resilience of quiescent eggs fundamentally depends on precise metabolic and molecular adaptations, particularly lipid metabolism and polyamine synthesis during quiescence (Prasad *et al.,* 2023). Notably, increased synthesis of polyamine is necessary for desiccation tolerance in *Aedes* eggs, as inhibition of this pathway can render eggs vulnerable to desiccation (Prasad *et al.,* 2023).

2.3.2 Reserve mobilisation

A dynamic balance of energy reserves is maintained by insects to support physiological functions, particularly during periods of stress and general activity. This section will briefly outline the current understanding of how reserves are mobilised.

Insects mobilise glycogen, a stored form of carbohydrate, in the form of trehalose, the levels of which are indicative of the nutritional status of the insects and thus likely trigger various metabolic regulatory pathways in the fat body (Lum and Chino, 1990; Thompson, 2003). Studies conducted on lepidopteran larvae and migratory locusts demonstrate that low trehalose concentration, typically associated with starvation or the non-feeding stage, is linked to increased lipid mobilisation (Beenakkers *et al.*,1985; Ziegler, 1991). Experimental injection of trehalose notably reduces the haemolymph lipid concentration, suggesting a shift in energy metabolism in times of urgency (Beenakkers *et al.,* 1985; Arrese *et al.,* 1996; Van der Horst *et al.,* 1997; Ziegler and Ibrahim, 2001). Haemolymph trehalose is also utilised by mosquitoes for foraging flights and swarming behaviours (Nayar and Van Handel, 1971). Additionally, under environmental stress, including dehydration, extreme temperatures and oxidative stress, stored glycogen is converted into trehalose and sugar alcohols, which are involved in protecting proteins and cellular membranes (Storey, 1997; Watanabe, 2002; Elben *et al.*, 2003).

On starvation, teneral adult mosquitoes respond by metabolising stored larval reserves, primarily lipids, as long as access to water is possible (Briegel, 1990a; Briegel, 1990b). Beyond prolonged starvation, lipids are strategically mobilised to sustain long-term flight via trehalose- and proline synthesis (Arrese and Soulages, 2010). Dynamic changes occur in female lipid levels during the gonotrophic cycle (Pinch *et al.*, 2021), during which a vast majority of maternal lipids are transferred to the ovaries and later to eggs (Briegel *et al.*, 2002). These lipids are mobilised during embryogenesis and for further sustenance of pharate larvae during quiescence (Arrese and Soulages, 2010).

Female energy reserves largely determine feeding choices and success (Ma and Roitberg, 2008). For instance, differential lipid reserves set the physiological state for host-seeking in mosquitoes (Renshaw *et al.*, 1995). Species-specific lipid threshold levels were proposed to be responsible for the differential initiation of blood seeking observed in two species of *Aedes* (Renshaw et al., 1995). Changes in climatic factors, including elevation in temperature or CO_2 level, or reduction in humidity, can alter energy reserves (Sasmita *et al.*, 2019; Hagan *et al.*, 2018; Paper I) or metabolism (Van Handel, 1966), affecting the feeding behaviour of disease-vectoring mosquitoes (Hagan *et al.,* 2018; Paper I). Such changes to resource-seeking behaviour warrant more investigation in the light of current anthropogenic climate change.

2.4 Mosquito olfaction

The sense of smell is vital for the survival of most insect species, and as with most insects, mosquitoes have a sophisticated olfactory system, which is adapted for detecting a myriad of volatile cues in the environment. While mosquito larvae rely on olfactory cues for foraging, this section will be biased towards adults, in line with the current literature. But for those interested in larval olfaction, relevant references are mentioned here (Clements, 1992; Riabinina *et al.*, 2016; Lutz *et al.*, 2017; Lutz *et al.*, 2019).

2.4.1 Structure and function of the olfactory system

The peripheral olfactory system of adult mosquitoes consists of the antennae, maxillary palps and proboscis (Konopka *et al.*, 2021; Wheelwright *et al.*, 2021). These olfactory tissues are covered by hair-like structures, sensilla, each hosting the dendrites of olfactory sensory neurons (OSNs), which translate the chemical signals into electrical signals (Wicher and Miazzi, 2021). Odour detection in insects begins with a series of “peri-receptor events”, in which volatile organic compounds (VOCs) enter the sensillum lymph through either pores or spokes of a sensillum, and move across the lymph, facilitated by chemosensory binding proteins, including odorant binding proteins (OBPs) and chemosensory proteins (CSPs). The OBPs and CSPs are synthesised and released by the sensillum support cells, *i.e.*, the thecogen, trichogen and tormogen cells, which ensheathe the OSNs

(Shanbhag *et al*., 2001; Larter *et al*., 2016; Prelic *et al*., 2022). The functional role of OBPs and CSPs is to bind, solubilise and transport the VOCs through the sensillum lymph to the chemosensory receptors residing in the dendrites of the OSNs (Vogt and Riddiford, 1981; Steinbrecht, 1996). Beyond their primary role in odorant transport, OBPs are also implicated in the modulation of the response of the OSN through gain control (Biessmann *et al*., 2010; Pelletier *et al*, 2010; Larter *et al*., 2016; Pelosi *et al*., 2018). The three-dimensional structure of OBPs and CSPs is constituted of six α-helical domains, forming a hydrophobic binding cavity, in which odorants are bound (Lartigue *et al*., 2002; Pelosi *et al*., 2018). The underlying mechanism for transport across the sensillum lymph has been proposed to be pH-induced conformational change in the three-dimensional structure, leading to the uptake of odorants at the entry pore/spoke of the sensillum, and their release near the dendritic membrane of the OSN (Leal *et al*., 2005; Zubkov *et al*., 2005; Manoharan *et al*., 2013).

The dendritic membrane of mosquito OSNs, as opposed to other insects, expresses one or a combination of chemosensory membrane-bound proteins belonging to up to three different families, including odorant receptors (ORs), ionotropic receptors (IRs) and gustatory receptors (GRs) (Younger *et al*., 2022; Adavi *et al*., 2024). The ORs and IRs function as ligand-gated ion channels by forming multimers with (a) highly conserved co-receptor unit(s) and a divergent ligand-selective tuning unit (Larsson *et al*., 2004; Abuin *et al*., 2011). Moreover, the highly conserved OR co-receptor (Orco) is required for the membrane trafficking of OR into the dendritic membrane (Benton *et al*., 2006). The OR-Orco complex, is proposed to be an asymmetric tetramer with a 1:3 stoichiometry of OR and Orco, with each having seven transmembrane domains (Wang *et al*., 2024; Zhao *et al*., 2024). On the other hand, the tuning IRs form heteromeric complexes with one or two highly conserved IR co-receptors, Ir25a, Ir76b and Ir8a (Benton *et al*., 2009; Abuin *et al*., 2011). Both olfactory receptor pathways are tuned to a wide repertoire of VOCs belonging to distinct chemical classes, in which ORs generally detect aldehydes, alcohols, ketones, aromatics, esters and terpenes (Hallem and Carlson 2006, Carey *et al*., 2010; Wang *et al*., 2010; Omondi *et al*., 2019; Pullmann-Lindsley *et al*., 2024), whereas IRs detect carboxylic acids and amines (Pitts *et al*. 2017; Raji *et al*., 2019). These chemical classes are emitted from a wide variety of resources used by mosquitoes, ranging from plants (Knudsen and Gershenzon, 2006), human or non-human hosts (Bernier, *et al*., 2008; De Obaldia *et al*. 2022; Zhao *et al*., 2022; Hinze *et al.,* 2022) to oviposition-sites (Afify and Galizia, 2015; Khan *et al.,* 2022), and mediate behavioural attraction and discrimination (DeGennaro *et al*., 2013; De Obaldia *et al*. 2022; Zhao *et al*., 2022). While ORs solely detect VOCs, the functional role of IRs also include taste-, hygro- and temperature-sensing (Wicher and Miazzi, 2021; Laursen *et al*., 2023; Morita *et al*., 2025). Similar to ORs, GRs form seven transmembrane ion channels (Frank *et al*. 2024; Gomes *et al*., 2024; Ma *et al*., 2024), which consist of various functional subfamilies, including sugar-, salt- and bitter-sensitive receptors (Sato *et al*., 2011; Kessler *et al*., 2013; Sanford *et al*., 2013; Sparks and Dickens, 2016), as well as the extensively studied CO_2-sensitive receptors (Kwon *et al*., 2007; Jones, *et al*. 2007 Lu *et al*., 2007; Erdelyan *et al*., 2012; McMeniman *et al*., 2014;; Robertson, 2019; Liu *et al*., 2020). The CO_2 detection system in the maxillary palps of mosquitoes consists of three highly conserved Grs, *i*.e., Gr1, Gr2 and Gr3 in culicines (Erdelyan *et al*., 2012; McMeniman *et al*., 2014) and Gr21, Gr22 and Gr23 in anophelines (Lu *et al*., 2007; Robertson, 2019; Liu *et al*., 2020), which form heterotrimers. While Gr1 and Gr3 have been demonstrated to be essential for the detection of CO_2 (Erdelyan *et al*., 2012; McMeniman *et al*., 2014; Kumar *et al*., 2020), Gr2 is proposed to function as a modulator (Kumar *et al*., 2020). Apart from CO_2, this Gr pathway is involved in the detection of acetone, a component of exhaled breath (Ghaninia *et al*., 2019; Younger *et al*., 2022). Additionally, many structurally diverse chemical compounds, though studied in high concentration, have been shown to activate or inhibit the response of CO_2-sensitive neurons (Tauxe *et al*., 2013). Whether other chemosensory receptors likely tuned to these VOCs are co-expressed in the CO_2-sensitive neurons remains unclear.

The response profile of an OSN depends on the specificity and sensitivity of the ligand-sensitive tuning receptor(s). In mosquitoes, the co-expression of ORs and IRs within a single OSN broadens the tuning breadth of each OSN (Adavi *et al*., 2024). However, this complicates the deconvolution of individual receptor contributions to odour detection, thereby hampering precise characterisation of receptor-specific tuning in vivo. Functional characterisation of heterologously and ectopically expressed ORs and IRs of fruit flies and mosquitoes suggest a variation in binding affinity of tuning receptors (Hallem *et al.*, 2004; Hallem and Carlson, 2006; Carey, *et al.*, 2010; Wang *et al.*, 2010). While broadly-tuned receptors are involved in the detection of overlapping chemical space in the environment, narrowly-tuned receptors play a critical role by detecting key chemical cues related to host, mating and danger (Stensmyr *et al.*, 2012; Andersson *et al.*, 2015), and are thus under positive selection pressure due to linked fitness benefits (Leary *et al.*, 2012). However, care must be taken when categorising receptors into broadly- or narrowly-tuned, as this is dependent on the heterologous expression system, concentration of the VOCs used and the number of compounds in the panel tested (Hallem *et al.*, 2004; Carey *et al.*, 2010; Wang *et al.*, 2010). In mosquitoes, specific ORs have been found to detect critical host or oviposition cues, including Or8 and its orthologs, which detect 1-octen-3-ol (Bohbot *et al.*, 2011), Or2, which detects indole (Xia *et al.*, 2008; Pelletier *et al.*, 2010) and Or10, which detects skatole (Xia *et al.*, 2008). In contrast to narrowly-tuned receptors, chemosensory receptors, with a broad tuning width, likely provide flexibility in detecting the vast odour space surrounding the insect (Andersson *et al.*, 2015).

Besides the canonical chemosensory receptors described above, the mosquito OSN membrane may also express sensory neuron membrane proteins (SNMPs) and noncanonical chemosensory receptors, including transient receptor potential (TRP) and pickpocket (PPK) ion channels (Matthews *et al.* 2016; Hill *et al.*, 2021). While SNMPs are involved in pheromone detection in Lepidoptera (Zhang *et al.*, 2020) and Diptera (Benton *et al.*, 2007; Zhang *et al.*, 2020; Cassau and Krieger, 2021), the functional role of SNMPs in mosquitoes remains unclear. In insects, TRP channels form transmembrane cation channels, with multiple sensory modalities, including thermo-, hygro-, mechano-sensation, as well as hearing and nociception (Liu *et al.*, 2007; Kang *et al.*, 2010; Kwon *et al.*, 2010; Fowler and Montell, 2013). Insect PPK ion channels are involved in the detection of environmental cues, including water, salts and odours (Liu *et al.*, 2003; Zhong *et al.*, 2010; Matthews *et al.*, 2019; Masague *et al.*, 2020), as well as in olfactory signal amplification in select OSNs (Ng *et al.*, 2019). The critical role that TRP and PPK channels play in assessing environmental signals calls for more research in characterising their functional role in mosquito sensory biology.

The chemosensory cues detected by membrane-bound receptors initiate a cascade of downstream signalling, leading to the generation of action potentials in each OSN (Sato and Touhara, 2008). At the periphery, timely termination of the olfactory signal transduction is required to ensure reliable coding of the temporal dynamics of the signal, and to enable free binding sites for a new set of VOCs and other chemosensory cues to be detected and for the OSNs to retain sensitivity (Leal, 2013). One way this is attained is by odorant degradation enzymes (ODEs), which include diverse protein families, including cytochrome P450 and carboxylesterases (Chertemps and Maïbèche, 2021), residing in the sensillum lymph. Excess odorants are trapped by ODEs and undergo enzymatic degradation to terminate the signal transduction pathway (Kaissling, 2001).

The olfactory signal initiated at the OSN dendrites is relayed to the primary olfactory processing centre, the antennal lobe (AL), in which OSN axons converge onto structural units, called glomeruli (Vosshall and Stocker, 2007). Glomeruli form functional units comprising OSNs, projection neurons (PNs) and local interneurons (LNs) (Ignell *et al*., 2005; Vosshall and Stocker, 2007), that process several features of the olfactory signal, including quality, quantity and temporal dynamics (Ignell *et al.,* 2010). The spatial arrangement of OSNs in the AL generates a chemotopic map with distinct combinatorial glomerular activity pattern for each VOC (Ghaninia *et al*., 2007; Galizia and Rössler, 2010; Zhao *et al*., 2022). The multi-glomerular LNs are involved in interglomerular signalling, mediating lateral inhibition to enhance odour discrimination (Seki *et al.,* 2017). From the AL glomeruli, the PNs carry information to the mushroom bodies (MBs) and lateral horn (LH) of the protocerebrum (Stocker *et al.,* 1997). The MBs are involved in learning, memory formation and contextualisation of the odour space (Heisenberg, 2003; Caron *et al.,* 2013), whereas the LH mediates innate responses to VOCs (Das Chakraborty and Sachse, 2021). The information processing at the LH is spatially organised to integrate odour valence and intensity (Strutz *et al.,* 2014; Das Chakraborty and Sachse, 2021). For insects, including mosquitoes, resource seeking is orchestrated through the integration of multimodal sensory cues, including olfactory, visual, thermal and gustatory at higher brain centres, including the MBs (Strausfield and Hildebrand, 1999) and LH (Galizia and Rössler, 2010; Strutz *et al.,* 2014; Thiagarajan and Sachse, 2022). Thus, timely convergence and processing of these signals are essential for the initiation of appropriate behavioural responses (McMeniman *et al.*, 2014).

2.5 Olfactory-guided behaviours

Mosquitoes display a wide repertoire of dynamic, temporally and spatially distinct olfactory-guided behaviours (Hill and Ignell, 2021). The foraging decisions made are pertinent to adult internal physiology, including nutritional status, age and mating, as well as external environmental changes (Hill and Ignell, 2021; Lahondère *et al.*, 2023). This section provides the context for one of the most important resource-seeking behaviours displayed by female mosquitoes, sugar seeking, as well as the dynamics involved, and the molecular determinants regulating this behaviour.

2.5.1 Sugar-seeking

Of the approximately 3700 mosquito species described worldwide, carbohydrate foraging is vital for both males and females (Clements, 1999), and adults of some species, including *Toxorhynchites* spp, are strictly nectarivorous (Collins and Blackwell, 2000). While males of all species are obligate nectar foragers, females of most species are “facultative” foragers, whose sugar foraging depends on physiological and environmental parameters (Gary and Foster, 2006; Fikrig *et al.*, 2020). The sugar-seeking activity of mosquitoes varies not only depending on the species, but also on the availability of other food resources (Yee and Foster,1992; Gary and Foster, 2006; Ma and Roitberg, 2008), and, in some cases, on geographical location (Olson *et al.*, 2020). Mosquitoes rely on sugars and secondary metabolites from plant sources, including floral nectars, extra floral nectaries and phloem, for flight energy, survival, reproduction and self-medication (Foster, 1995; Foster, 2022). Beyond sugar, floral nectar also contains amino acids, salts and vitamins (Kevan, 1983; Nicolson, 2007; Baker and Baker, 1983), which are metabolised and play a significant role in regulating mosquito physiology (Rivera-Perez *et al.*, 2017).

The investment in sugar-seeking relies on the energy demands of the female (Yee and Foster, 1992; Ma and Roitberg, 2008). On emergence, adults are deprived of carbohydrate and lipid reserves, and feeding on plant sugars can replenish these reserves (Foster, 1995; Briegel *et al.*, 2001).

Teneral females are attracted to floral odours, while with maturation, females develop the competence to host seek to obtain a blood meal (Foster and Takken, 2004; Omondi *et al*., 2019; Tallon *et al*., 2019). Mating and other physiological changes, associated with each gonotrophic cycle, affect floral seeking, which is reflected in the avidity of females to sugar feed (Gary and Foster, 2006; Stone *et al*., 2011; Christ *et al*., 2017; Tenywa *et al*., 2024; Paper III). In general, mating shifts the nutritional demands in female insects, redirecting their resource-seeking behaviour for reproductive success (Carvalho *et al*., 2006). Similarly, in aging female mosquitoes, sugar foraging gradually decreases, owing to the shift in physiological requirements of a female to blood feed for egg development (Clements, 1999). Moreover, following ingestion of a blood meal, there is a complete inhibition of sugar seeking (Stone *et al*., 2011; Christ *et al*., 2017). However, the avidity towards nectar sources is regained in gravid females (Klowden, 1986), and post-oviposition before the next blood meal (Gary and Foster, 2006).

Besides the internal physiology of the mosquito, the external environment influences the sugar-foraging decisions. The availability and quality of suitable nectar sources are prone to seasonal changes, as well as affected by human interventions (Foster, 1995; Barredo and DeGennaro, 2020), thus influencing the mosquito feeding dynamics (Martinez-Ibarra *et al*., 1997; Fikrig *et al*., 2020). Furthermore, an increase in atmospheric temperature has been shown to interact with sugar-feeding behaviour in modulating the diel activity pattern, with access to sugar improving the survival of *Ae. aegypti* at higher temperatures (Upshur *et al*., 2019). How other climatic factors related to anthropogenic climate change, such as elevation in CO_2, ozone and other pollutants, influence the nectar-seeking choices needs further investigation.

2.5.2 Plant preferences

Mosquitoes display differential preferences for plants, predominantly dependent on the availability of sugars (Foster, 1995; Foster, 2022). While mosquitoes prefer sugar sources that provide the maximum energy payoff and maximise fitness (Manda *et al*., 2007; Gouagna *et al*., 2014), this is not always the case (Pare *et al*., 2024), indicating that both intrinsic and extrinsic factors influence the host-plant preference of mosquitoes. However, it is hypothesised that mosquito plant preference is predominantly innate, with plasticity allowing adaptation to the immediate environment through experience (Foster 1995; Barredo and DeGennaro 2020). A sugar-foraging mosquito is guided by multiple sensory modalities, including floral VOCs, CO_2 and visual cues, which synergise to enhance the attraction of mosquitoes (Nyasembe *et al*., 2012; Peach *et al*., 2019). The plethora of volatile secondary metabolites, or VOCs, produced by plants is often indicative of the availability and nutritional value of the source (Goff and Klee, 2006).

The hierarchy of mosquito plant preferences has been studied through various semi-field and laboratory assays, including visual scoring of feeding, testing the presence of sugar by either anthrone test or analytical chemistry, assessing attraction to plants and their volatile extracts and, in the last decade, through DNA barcoding of ingested plant material (Foster, 1995; Nyasembe *et al*., 2018; Foster 2022). Earlier semi-field studies on the mosquito plant ecology demonstrated both broad and narrow plant preferences across *Aedes* (Muller *et al*., 2011; Sissoko *et al*., 2019; Upshur *et al*., 2023), *Anopheles* (Impoinvil *et al*., 2004; Manda *et al*., 2007; Gouagna *et al*., 2014) and *Culex* species (Chen and Kearney, 2015). However, limited inferences were made on the availability of corresponding plant sources in the local environment, across seasons and, in other cases, the precise identification of the plant species (Foster, 1995). DNA barcoding resolves this limitation by identifying the specific plant species in field-collected mosquitoes (Nyasembe *et al*., 2018). Further assessment of headspace volatiles and behavioural responses to these plants has enabled identification of bioactive VOCs that guide mosquitoes to plant sources (Nyasembe *et al*., 2018; Upshur *et al*., 2023).

While VOCs emanate from both floral and vegetative parts of the plant, little is known about how the mosquito perceives these signals and the relative significance of different VOCs in mediating attraction. Headspace volatile profiles of preferred host plants reveal a diverse array of VOCs belonging to different chemical classes, predominantly terpenoids, aldehydes and aromatics (Otienoburu *et al*., 2012; Nyasembe *et al*., 2012; Nikbakhtzadeh *et al*., 2014; Nyasembe and Torto, 2014, Nyasembe *et al*., 2018, Upshur *et al*. 2023; Omondi and Wondwosen *et al.,* in review), which are the most abundant in majority of floral scents (Knudsen and Gershenzon, 2006). Mosquitoes detect only a subset of these floral VOCs, evident from combined chemical and electrophysiological analyses, revealing the response profile of individual OSNs (Wondwosen *et al.,* in prep) and selective tuning of ORs (Zeng *et al*., 2019; Pullmann-Lindsley *et al*., 2024; Vainer *et al*., 2024; Omondi and Wondwosen *et al.,* in review; Paper III). While different mosquito species detect different constituents of floral scent, likely due to differences in tuning of expressed ORs, they retain similar behavioural preferences (Omondi and Wondwosen *et al.,* in review; Wondwosen *et al.,* in prep). The shared detection of VOCs across evolutionarily divergent mosquito species suggests their role as conserved, generalist cues for plant location (Nyasembe *et al*., 2018; Wondwosen *et al.,* in prep). Species-specific bioactive VOCs, on the other hand, may facilitate discrimination between suitable plant sources (Nyasembe *et al*., 2018; Wondwosen *et al.,* in review). Notably, numerous bioactive VOCs overlap between floral-, host- and oviposition-site-related resources, indicating the concept of chemical parsimony (Blum, 1996; Ignell and Hill 2020). Chemical parsimony refers to an insect's adaptive and efficient use of a finite number of VOCs in multiple ecological and behavioural contexts, as well as the restrictive biosynthesis pathways involved for the production of VOCs (Blum, 1996). These generic VOCs, detected through a few conserved olfactory receptors, and the more specific VOCs, detected by many species-specific olfactory receptors, may guide mosquito attraction and discrimination of sugar sources (Hill and Ignell, 2021).

Floral VOCs mediate the observed discrimination between sugar sources in both semi-field (Müller *et al*., 2010; Sissoko *et al*., 2019) and laboratory assays (Nyasembe *et al*., 2012; Nikbakhtzadeh *et al*., 2014; Wondwosen *et al.,* in prep). As such, these studies demonstrate the importance of blend composition and ratio of VOCs within the blend (Ignell and Hill 2020; Hill and Ignell 2021). For example, nonanal and lilac aldehyde mediate the differential attraction of *Aedes* species to different *Platanthera* orchid species, thereby facilitating pollination of these orchids (Lahondere, *et al*., 2020). Furthermore, subtractive blends, in which individual VOCs are omitted, provide support for blend composition and the relative significance of individual VOCs in mosquito sugar-seeking (Otienoburu *et al*., 2012; Paper III). In laboratory and field assays, synthetic odour blends, containing bioactive VOCs in their natural ratio, are an efficient way to assess attraction and preference (Nyasembe *et al*., 2012; Wondwosen *et al*., 2016, Wondwosen *et al*., 2017; Wondwosen *et al*., 2018), and expand the use of plant/floral odour-baited traps for vector control (Nyasembe *et al*., 2014).

Other sensory modalities, including visual cues and CO_2, which have been mostly studied in the context of host seeking, also influence floral-seeking decisions (Peach *et al.*, 2019). The plant-released CO_2 is used by foraging insects for locating nectar sources (Goyret *et al.,* 2007; Guerenstein and Hildebrand, 2008), and as a reliable indicator of nectar abundance (Thom *et al.,* 2004; Guerenstein *et al.,* 2004; Omondi and Wondwosen *et al.,* in review). For example, in female *Anopheles* mosquitoes, the addition of CO_2 to a synthetic floral odour blend has a synergistic and contextual effect on attraction (Omondi and Wondwosen *et al.,* in review). Moreover, in *Aedes* and other mosquito species, the addition of CO_2 and visual cues, including floral colour and shape (Sandholm and Price, 1962; Magnarelli, 1977; Magnarelli, 1979), modulate mosquito attraction to floral inflorescences (Peach *et al.*, 2019).

2.5.3 The link between floral-seeking and host-seeking

Both sugar and blood meals significantly shape the life history traits of females, and their preference for either resource is modulated by internal energy demands (Stone and Foster, 2013). Furthermore, the diel activity period for floral seeking often overlaps with host-seeking, emphasising the intertwined relationship between these two foraging decisions (Sissoko *et al.*, 2019). In females, there are parallel pathway for sugar- and blood meal involving distinct appendages and digestive tract (Gordon and Lumsden, 1939; Jove *et al.*, 2020). Imbibed sugar meals are targeted to the crop, whereas blood is directed to the midgut (Gordon and Lumsden, 1939). This intricate behavioural interplay has evolutionary roots. Haematophagy is believed to have emerged independently numerous times among arthropods (Lehane, 2005). One prominent hypothesis proposes that blood-feeding mosquitoes evolved from ancestral phytophagous forms that possessed piercing or sucking mouth parts, originally evolved for feeding on either plants or insects (Peach and Gries, 2019). The transition from plant feeding to blood feeding is proposed to have been facilitated by chemical parsimony, *i.e.*, shared VOCs between plant- and host-related resources (Peach and Matthews, 2022). Over evolutionary time, female mosquitoes evolved a dynamic relationship between sugar and host-seeking, shaped by both ecological pressures and resource availability.

The evolutionary flexibility to feed on plant- and host-related resources is evident in anthropophilic mosquito species, such as *Ae. aegypti* and *An. gambiae*, which can rely almost exclusively on blood-derived nutrients to meet their reproductive and metabolic demands (Beier, 1996; Harrington *et al*., 2001). While *Ae. aegypti* females may survive exclusively on human blood, this strategy compromises longevity despite enhanced reproductive output (Harrington *et al*., 2001; Braks *et al*., 2006), and such a strategy is adaptive in environments with consistent access to blood hosts (Scott and Takken, 2012). Anthropophilic mosquitoes do, however, retain an inherent attraction to floral VOCs (Foster and Takken, 2004) and frequently visit plant sources throughout their gonotrophic cycle to replenish their energy reserves and maintain reproductive output (Hancock and Foster, 1993; Foster, 1995; Fernandes and Briegel, 2005; Stone *et al*., 2011; Sissoko *et al*., 2019; Olson *et al*., 2020). Thus, for anthropophilic species, the proximity of human hosts and the presence of sugar sources may significantly influence the choice of sugar or blood meal (Stone and Foster, 2013).

Sugar deprivation has profound consequences for mosquito biology, influencing survival, insemination rates, blood-feeding frequency and reproductive fitness (Stone and Foster, 2013). In addition to a reduced number of mature eggs, sugar-deprived smaller gravid females (Briegel, 1985; Briegel,1990; Briegel *et al*., 2001) are burdened with energy demands that warrants additional blood meals before oviposition to meet both somatic and reproductive energy demands (Klowden, 1986; Hancock and Foster, 1993). The presence of sugar in the crop can physically restrict the volume of blood meal, due to limited abdominal space in females (Stone and Foster, 2013). Thus, sugar deprivation in females increases the blood meal volume imbibed and the frequency of biting (Fernandes and Briegel, 2005). Sugar feeding is also essential for both males and females to sustain flight energy expenditure, in the absence of which mating success, dispersal and foraging capabilities may be affected (Nayar and Van Handel, 1971; Spitzen and Takken, 2018). Sugar deprivation in males can, furthermore, reduce insemination performance and shorten the lifespan (Stone *et al*., 2009). Male nutritional status further exerts an indirect influence on female physiology and foraging choices (Villarreal *et al*., 2018; Huck *et al*., 2021). Male diet, *i.e.*, plant sugars, can also affect the overall functioning of the male accessory glands (MAGs) (Baldini *et al*., 2012; Huck *et al*., 2021), which play a vital role in regulating female reproductive physiology via the proteins transferred to females during mating (Fernandez and Klowden, 1995; Klowden, 1999; Villarreal *et al.*, 2018). The nutritional stress in males can influence female reproductive output by altering resource allocation and ovarian physiology (Gillot, 2003; Clifton *et al.*, 2014). The overall effect of sugar feeding on adult survival, biting rates and reproduction may have a significant effect on the overall population dynamics and vectorial capacity (Ferguson *et al.*, 2010; Stone and Foster, 2013).

2.6 Modulation of olfactory-guided behaviours

The transition from one behaviour to another across temporal and spatial scales requires a plastic sensory system (Gadenne *et al.*, 2016). Insect behaviour is modulated by internal physiology, as well as the external environment. This section discusses modulatory changes occurring at the peripheral olfactory system to higher brain centres.

2.6.1 Regulation of chemosensory gene expression

Regulation at the peripheral chemosensory system level is one the most common cost-efficient ways an insect can adapt to a changing environment over different time scales (Bruce and Pickett, 2011; Hill and Ignell, 2021). Over longer evolutionary time scales, these changes include a change in OSN sensitivity or tuning, the number of OSNs and a complete loss or gain of an OSN type (Zhao and McBride, 2020). In contrast, over shorter time scales, transcriptional regulation of chemosensory gene expression may modify the OSN response profile (Zhao and McBride, 2020). In this section, the effect of intrinsic and extrinsic factors on the regulation of chemosensory gene expression is discussed.

In holometabolous insects, such as mosquitoes, a vast majority of larval OSNs are eliminated during metamorphosis, with adult neuronal circuits emerging and maturing following pupation (Barish and Volkan, 2015; Yan *et al.*, 2020). The OSN-specific olfactory receptor expression has historically been considered developmentally pre-determined, governed by a set of specific transcription factors and cis-regulatory elements located upstream of the chemosensory receptor genes, exhibiting no flexibility for post-developmental modulation (Jafari and Alenius, 2015; Mika and Benton, 2021). However, recent studies in *D. melanogaster* and mosquitoes reveal that the developmental window for chemosensory receptor gene expression remains permissive into the early adult stage, facilitated by chromatin state regulation and post-transcriptional receptor feedback (Jafari and Alenius, 2015; Jafari *et al*., 2021). Growing evidence highlights that internal physiology and external environmental changes, during later stages of the adult insect life, can also modulate the expression of chemosensory genes (Gadenne *et al*., 2016).

2.6.2 Role of the external environment

Environmental changes, including increases in temperature, CO_2 and ozone levels, can modulate insect behaviour and modify intra- and interspecies chemical communication (Boullis *et al.,* 2016; Vanderplanck *et al*., 2021; Knaden *et al*., 2022; Baleba *et al*., 2023; Jiang *et al*., 2024). For instance, temperature fluctuations can alter the volatility and concentration of VOCs (Zhou *et al*., 2017), while elevated ozone levels can modify the odour space by oxidation of double bonds (Knaden *et al.,* 2022). While the impact of temperature and ozone has gained much attention with climate change, the impact of elevation in CO_2, another major driver of anthropogenic climate change, on insect chemical communication seems comparatively under-explored. However, current evidence highlights how elevation in CO_2 can affect insect chemical communication indirectly *via* altered plant biochemistry (Zavala *et al*., 2017) and directly by altered sensory function (Sun and Ge, 2011; Majeed *et al*., 2014; Paper II). Notably, the changes in the environment are reflected in insect olfactory circuits (Riveron *et al*., 2009; Martin *et al*., 2011) and at the molecular level (Riveron *et al*., 2013; Paper II).

Transcriptome analyses of olfactory appendages demonstrate that environmental changes, including abiotic stressors, such as insecticides, VOCs, CO_2 and temperature can modulate the olfactory machinery at the molecular level (Low *et al*., 2010; Riveron *et al*., 2013; Li *et al*., 2017; Li, *et al*., 2019; Yang *et al*., 2021; Zhang *et al*., 2022; Guo *et al.,* 2025; Paper II). However, the underlying mechanisms governing these responses remain poorly understood. While such stressors are known to broadly induce transcription of xenobiotic response genes (Mack and Attardo, 2023), evidence suggests that stressors may also exert specific and localised effects within the olfactory system, though this requires further studies (Riveron *et al*., 2013; Wang *et al*., 2023; Guo *et al.,* 2025; Paper II).

Xenobiotic response genes, which are part of the insect detoxification system, have been studied in mosquitoes and other insect pests, predominantly in reference to the adaptation to insecticides (Poupardin *et al*., 2008; Poupardin *et al*., 2010; Nkya *et al*., 2013; Gao *et al*., 2022). These genes are broadly expressed and include OBPs and proteins belonging to the cytochrome P450 (CYPs), insect cuticle proteins, glutathione-S-transferase (GSTs), glucuronosyl transferases (GTs) and carboxyl/cholinesterases (CCEs). Many of the xenobiotic response genes function as ODEs (Leal *et al*., 2013; Wu and Hoy, 2016; Balabanidou *et al*., 2018; Lu *et al*., 2021; Abendroth *et al*., 2023) and ensure timely clearing of the sensillum lymph to ensure detection of relevant VOCs.

Exposure to noxious concentrations of VOCs in the environment requires rapid clearance of the sensillum lymph to protect OSNs from damage, maintain sensitivity and prevent overstimulation (Dalton, 2000; Mappin *et al*., 2023). Prolonged exposure to a VOC can drive adaptation of the OSN, triggering differential regulation of chemosensory gene expression, including ORs, OBPs and ODEs (Koerte *et al*., 2018; Baldwin *et al*., 2021; Mappin *et al*., 2023). Furthermore, such prolonged exposure to a VOC is associated with alterations at the OSN level, including secondary messenger gene expression (Deshpande *et al.,* 2000; Jafari and Alenius, 2021; Iyengar *et al.,* 2010), as well as modulatory and morphological changes at higher brain centres (Devaud *et al.,* 2003; Fabian and Sachse, 2023). All these adaptations may lead to altered detection and perception of the external environment by the insect.

Carbon dioxide (CO_2) is an important constituent of an insect odourscape, signalling resource location (Guerenstein and Hildebrand, 2008). Anthropogenic increases in CO_2 concentrations affect the odour-mediated behaviours of insects, including host-, mate-, oviposition-site-seeking and alarm sensing (Stange *et al*., 1997; Sun *et al.,* 2010; Majeed *et al*., 2014; Boullis *et al*., 2016; Choi *et al.,* 2018). While in mosquitoes and some moth species, this negative impact is mediated through sensory constraints on the CO_2 sensory system (Stange, 1997; Stange and Wong, 1993; Majeed *et al*., 2014), implications of the interaction of elevated CO_2 with the central nervous system have been reported (Sun *et al.,* 2010; Choi *et al.,* 2018). For instance, in *Helicoverpa armigera,* males display a CO_2 concentration-dependent reduction in receptivity to sex pheromones, where CO_2-mediated modulation at the AL was proposed as a possible mechanism (Choi *et al.,* 2018). Additionally, in aphids reared under elevated CO_2 levels, changes to the activity of acetylcholinesterase were proposed to mediate the reduced escape behaviours (Sun *et al.,* 2010). The underlying mechanism of elevated CO_2 affecting these behaviours remains inconclusive. However, environmental changes in CO_2 concentration seem to have a broad effect on the molecular gene expression level (Helenius *et al.,* 2009; Paper II).

Temperature is a key environmental stressor affecting both odour detection and odour-guided behaviour across insect species, including fruit flies, honeybees and mosquitoes (Riveron *et al*., 2009; Martin *et al*., 2011; Baleba *et al*., 2023; Lahondere *et al*., 2023; Guo *et al*., 2025). Besides altered OSN firing properties, temperature modulates chemosensory gene expression, affecting both peri-receptor events and the function of OSNs through OBP and membrane-bound receptor gene regulation, respectively (Riveron *et al*., 2013; Guo *et al*., 2025). Moreover, the regulatory mechanism underlying chemosensory receptor expression, including transcriptional factors and chromatin state regulation during early adult life, is sensitive to environmental stress, including temperature as well as nutrient deprivation (Jafari and Alenius, 2015, Gonzalez *et al*., 2019; Jafari *et al*., 2021), highlighting stress-induced plasticity in the peripheral olfactory system.

2.6.3 Internal-state dependent plasticity

Throughout the lifetime of a mosquito, across adult maturation and aging, the peripheral olfactory system undergoes changes in sensitivity and specificity reflecting the dynamic shifts in behaviour (Hill and Ignell, 2021). Across insect species, this physiological plasticity is correlated with modulation of the olfactory behavioural responses to different resource cues (Gadenne *et al.,* 2016; Anton and Rossler, 2021). The following section will cover how the internal physiology of the female, including age, mating and feeding state, modulates the behavioural and physiological phenotype, as well as chemosensory gene expression.

Newly-emerged female mosquitoes undergo a brief behavioural refractory period of resting when the peripheral olfactory system undergoes maturation, correlated with the expression of members of the major chemosensory gene families (Davis, 1984a; Foster and Takken, 2004; Hill and Ignell, 2021). Following 24 h post-emergence, females display age-dependent floral- and host-seeking (Foster, 1995; Omondi *et al.,* 2019; Tallon *et al.,* 2019; Hill *et al.,* 2021; Paper III). The first meal imbibed by a female is generally a sugar-rich floral nectar meal to replenish their energy reserves (Foster, 1995; see 2.5.1 Sugar seeking). Teneral females are more responsive to floral odour compared to older females, which correlates with an altered perception of floral VOCs with aging (Foster and Takken, 2004; Omondi and Wondwosen *et al*., in review; Paper III). While teneral females are able to detect host odours, the behavioural response is often aversive (Omondi *et al*., 2019; Tallon *et al*., 2019). Females possess several ORs narrowly tuned to floral VOCs (Zeng *et al*., 2019; Pullmann-Lindsley *et al*., 2024; Vainer *et al*., 2024; Paper III). However, the temporal dynamics of these Ors' expression remain largely unknown, except for a few, such as *Or117* in *Ae. aegypti*, the gene transcript abundance of which decreases with age (Tallon *et al*., 2019; Hill and Ignell, 2021; Paper III).

As females age, they gradually shift to host seeking for a blood meal (Hill and Ignell, 2021), during which they become more sensitive to host cues, such as CO_2 and other salient host VOCs (Davis, 1984; Grant and O'Connell, 2007; Omondi *et al.,* 2015; Omondi *et al.,* 2019). This behavioural shift correlates with a concerted upregulation of chemosensory gene transcript abundance, including ORs, IRs and OBPs (Omondi *et al*. 2015; Omondi *et al*., 2019; Tallon *et al*., 2019; Hill and Ignell 2021). While the majority of chemosensory genes follow this age-dependent expression pattern, a few exceptions exist, such as *Or39* in *An. coluzzii* (Omondi, Ghaninia *et al*. 2019). These receptors have been proposed to act as molecular switches that regulate female resource-seeking (Hill and Ignell 2021). Targeted gene knockout studies performed in *Anopheles* (Hinze *et al.,* 2023) and *Aedes* (Paper III) provide insights into the causative molecular mechanisms underlying mosquito behaviour.

While there is no distinction in timeline between sexual and chemosensory system maturation, in *Ae. aegypti*, males and females attain sexual maturity within 24 h post-emergence (Sanchez *et al.,* 2023). Once males of *Ae. aegypti* attain sexual maturity, they take part in multiple inseminations (Jones, 1968; Alfonso-Parra *et al.,* 2014), whereas mature females mate only once (Clements, 1999; Carvalho *et al.,* 2018), with successful mating ensuring refractoriness to subsequent mating (Duvall *et al.,* 2017). In numerous insects, including mosquitoes, mating induces physiological and behavioural changes in females (Jones, 1981; Gadenne *et al.,* 2016; League *et al.,* 2021). During mating, in addition to sperm, males transfer male accessory gland-secretory proteins to the female, which enforces paternity, and causes physiological and behavioural changes in females (Naccarati *et al.,* 2012; Duvall *et al.,* 2017). In *Ae. aegypti* females, mating can modulate flight activity (Jones, 1981), increase blood feeding (Villareal *et al.,* 2018; League *et al*., 2021), as well as enhance longevity and reproductive output (Helinski and Harrington, 2011). These changes are correlated with transcriptomic changes in the female reproductive tract and peripheral olfactory system (Alfonso-Parra *et al.,* 2016; Alonso *et al.,* 2019). During courtship, there is differential regulation of many chemosensory gene expression in females, particularly *OBPs,* that are downregulated on male contact and later upregulated following mating, suggestive of an intermediary state facilitating mating success (Alonso *et al*., 2019). Mating modulates host-seeking decisions, depending on the nutritional status of the female (Klowden and Lea, 1979) and stimulates egg production (Baldini *et al.,* 2012). Notably, mating is not a prerequisite for blood feeding and can occur before or after a blood meal (Mayilsamy *et al.,* 2021; Sanchez *et al.,* 2023). Mating in gravid females reduces the behavioural response to host stimuli, likely via seminal fluid proteins, a mechanism that requires further investigation (Lee and Klowden, 1999; Naccarati *et al*., 2012; Duvall *et al*., 2017).

While the onset of host-seeking is generally achieved 4-to-5 days post-emergence, the dynamics of floral- and host-seeking may vary depending on energy demands and resource availability (Foster, 1995; Foster, 2022). However, other physiological changes, including mating or a blood feeding, may occur during this age window and can interact to modulate the expression of several chemosensory gene families at the periphery correlated with changes in physiological and behavioural responses to resource cues (Hill and Ignell, 2021; League *et al.,* 2021).

Host-seeking in female mosquitoes is governed by a complex interplay between external environmental cues and internal physiological states (Tung and Fonseca, 2024). Following ingestion of a blood meal, host seeking is rapidly suppressed until oviposition is completed (Klowden and Lea, 1979a;

Klowden and Lea, 1979b; Davies, 1984b; Brown *et al.,* 1994; Takken *et al.,* 2001; Qiu *et al.,* 2006a; Hill *et al.,* 2021). This suppression is biphasic: an immediate phase mediated by mechanosensory input from abdominal stretch receptors (Klowden and Lea, 1979a), and a longer suppression period involving the activation of neuropeptide Y-like receptor 7 (NPYLR7), likely triggered by signals associated with oogenesis (Liesch *et al*., 2013; Duvall *et al.,* 2019).

A blood meal induces time-dependent transcriptional regulation of several chemosensory gene families, including *ORs*, *IRs*, *OBPs* and *CSPs* (Rinker *et al*. 2013; Taparia *et al*. 2017), likely via neuromodulatory peptide signalling (Klowden and Lea, 1979; Liesch *et al*., 2013; Christ *et al*., 2017; Duvall *et al*., 2019). Notably, the antennal abundance of *Ir75k,* an ionotropic receptor tuned to short-chain carboxylic acids, a key oviposition site cue (Chen *et al*., 2019), increases post-blood meal and coincides with a heightened OSN sensitivity to these ligands, marking a shift from host- to oviposition-site-seeking (Hill *et al*., 2021). Moreover, the integration of *OR* gene transcript abundance with receptor tuning profiles (Carey *et al*., 2010) suggests that antennal detection capacity is dynamically restructured to support the behavioural transition from host- to oviposition-site-seeking (Rinker *et al*., 2013). Sugar meal ingestion also transiently suppresses host seeking, albeit over a shorter duration (Foster, 1995; Christ *et al.,* 2017). However, it remains unknown whether sugar feeding induces transcriptional changes in chemosensory genes akin to those observed post-blood meal. Upon oviposition, females fully resume resource-seeking behaviours to replenish energetic reserves through either a subsequent sugar or blood meal (Foster, 1995). The cyclical modulation of chemosensory gene expression across the gonotrophic cycle likely provides females with the sensory plasticity required to alternate efficiently between floral-seeking, host-seeking and oviposition-site-seeking (Hill and Ignell 2021; Hill *et al*., 2021).

2.6.4 Regulation at higher brain centres

Modulation of the central olfactory system has been extensively studied across insect species, demonstrating significant responsiveness to internal state and external environmental cues (Gadenne *et al*., 2016; Anton and Rossler, 2021). Only recently, *Ae. aegypti* is emerging as a model organism for studying the neural basis of behaviours in mosquitoes (Weiss and

McBride, 2024), and for this reason, very little is currently known about the link between the extensively studied peripheral olfactory system to the modulation at higher brain centres. However, a few cases of interest related to resource-seeking are discussed below.

Within the antennal lobe, extrinsic neurons and local interneurons express a variety of biogenic amines and neuropeptides, which are implicated in olfactory modulation (Siju *et al.* 2008; Siju, 2009; Siju *et al.*, 2014). In *Ae. aegypti*, ingestion of blood and/ or sugar differentially affects the levels of neuropeptides in the antennal lobe (Christ *et al.*, 2017). Notably, the artificial injection of a binary mixture of neuropeptides is sufficient to suppress host-seeking behaviour in females, suggesting a direct neuromodulatory role in the regulation of olfactory-guided behaviours (Christ *et al.*, 2017; Christ *et al.*, 2018).

Experience-driven modulation of mosquito host preference has also been recognised as a factor influencing behavioural plasticity (Vinauger *et al.*, 2014; Vinauger *et al.*, 2016). Prior feeding experience can override innate host preferences, suggesting that associative learning might play a role in shaping mosquito foraging behaviours (Vantaux *et al.*, 2014). Moreover, mosquitoes can associate host- and plant-related volatiles in an aversive conditioning paradigm (Wolff *et al.*, 2023). However, the extent of learnability is determined by multiple factors, including species, the ecological context, the innate valence of the odorant and dopaminergic innervations of the antennal lobe glomeruli (Wolff *et al.*, 2023). For instance, mutagenesis of the dopamine-1 receptor, and pharmacological inhibition using dopamine receptor antagonists, both abolish learning in *Ae. aegypti* and *Anopheles stephensi* females, emphasising the role of dopamine signalling in olfactory learning and memory formation (Vinauger *et al.*, 2018; Wolff *et al.*, 2023).

3. Aim and objectives

The overall aim of this thesis was to investigate how environmental factors, as a result of climate change, and intrinsic factors modulate *Aedes aegypti* physiology and behaviour.

The first objective was to determine the effect of predicted elevated CO_2 and extended egg quiescence period on *Aedes aegypti* life-history traits (**Paper I**).

The second objective was to assess the effect of two environmental stressors, elevated CO_2 and extended egg quiescence period on the peripheral olfactory system of *Aedes aegypti* (**Paper II).**

The third objective was to elucidate the role of odorant receptor, *Or117* in modulating the age- and mating-dependent regulation of floral seeking in *Aedes aegypti* (**Paper III**).

4. Methodology

This chapter outlines the methodologies employed to generate the results presented in Papers I-III. While additional methods, including electrophysiology, functional characterisation of receptor and mutagenesis, are discussed in the respective manuscripts, they are not included in this section. For comprehensive details, readers are kindly referred to the individual papers.

4.1 Maintenance of *Ae. aegypti* colonies

Colonies of all *Ae. aegypti* genotypes used for the experiments (Paper I-III) were maintained at 27 ± 2 °C and 65 ± 5% relative humidity, and a 12 h: 12 h light: dark cycle. Larvae were reared in plastic trays (23.5 cm × 18 cm × 7.5 cm; filled with 600 ml water) and fed with fish food (TetraMin® Flakes, Melle, Germany). The emerging adults had *ad libitum* access to 10% sucrose solution and older females were given access to sheep blood (Håtunalab AB, Bro, Sweden) through a collagen membrane and a membrane feeding system (Hemotek Ltd, Blackburn, UK). Later, blood-fed females were presented with a wet filter paper atop a water-filled 30 mL cup (Nolato-Hertila, Åstorp, Sweden) for oviposition for 48 h. Collected eggs were subsequently dried, labelled and stored in containers for further experimental use (Papers I and II). Eggs were categorised as 'new' or 'older' based on their quiescent duration: two weeks for new eggs and 3–6 months for older eggs (Papers I and II). In all experimental setups, newly-emerged females (24 h-to-36 h post-emergence) had access to water only, while five-day-old females (120 h-to-132 h post-emergence) were maintained on a 10% sucrose solution for four days before being deprived of sucrose for 24 h prior to the experiments (Paper III).

4.2 Rearing of *Ae. aegypti* in CO_2 climate chambers

Experiments assessing the impact of elevated CO_2 and extended egg quiescence duration on *Ae. aegypti* life history traits and peripheral olfactory system were conducted in high-precision climate chambers at SLU, Alnarp campus (ca 11.5 m^2 with a free height of 2.3 m), maintained at 27 ± 2 °C and 65 ± 5% relative humidity, and a 12 h: 12 h light: dark cycle. The CO_2 level in the three chambers was maintained at ambient (~ 420 ppm, Paper I and II), 600 ppm (Paper I) and 1000 ppm (Paper I and II). A filter paper containing age-controlled eggs was divided equally across the three climate chambers. The eggs were placed in larval trays supplemented with a small quantity of fish food (TetraMin® Flakes) to induce hatching. After 18 h, hatched pharate larvae were counted and transferred to larval trays at a density of 100 larvae per 600 mL of water. Larvae were fed fish food (1 mg/larva) daily to maximise life-history trait parameters and minimise competition (Arrivilaaga and Barrera, 2006). Water was replaced every second day to control for microbial growth and accumulation of debris (Papers I and II).

4.3 Assessing the life history traits of immature and adult stages

4.3.1 Larval survival and developmental duration

To investigate the impact of elevated CO_2 and extended egg quiescence duration on larval growth and survival, in each climate chamber, larvae were monitored for survival probability and developmental duration. Survival was assessed every 12 h by counting live individuals until all larvae either pupated or died. Developmental duration was determined by recording pupation time at 12 h intervals. Pupae were transferred to small plastic cups filled with distilled water and subsequently labelled and placed in BugDorm cages (17.5 cm × 17.5 cm × 17.5 cm; Megaview Science Co., Ltd, Taichung, Taiwan) for adult emergence.

For investigating the underlying mode of action of elevation in CO_2 on larval life history traits, artificially-induced acidity experiments were performed on larvae emerging from 2-week-old eggs. The pH of larval water was artificially decreased to levels measured in larval water acclimatised to 1000 ppm CO_2 level, by addition of 0.1 mL 0.1 N hydrochloric acid (Weiss *et al.*, 2018). The pH of larval water was monitored, and larval survival and developmental duration assessed as mentioned above.

4.3.2 Adult survival, body size and fecundity

Following emergence, adult mosquitoes were provided access to only distilled water, and mortality recorded every 12 h until all mosquitoes in a cage were deceased. For each experimental cage, no more than 50 individuals were kept to ensure resource availability and reduce competition. Adult body size was measured by dissecting the right wing and measuring the length from the axillary incision to the apical margin (excluding the fringe) using an ocular micrometre (Bickley, 1981).

4.3.1 Estimation of adult teneral energy reserves

The teneral metabolic reserves accumulated by females in response to CO_2 level and egg quiescent duration were analysed by quantifying the carbohydrate, glycogen, lipid and protein content. For the analysis, individual adults (up to 12 h post-emergence) were freeze-killed and stored in 2 mL Eppendorf tubes at -20 °C. The biochemical quantification followed Van Handel's calorimetric estimation methods, as modified by Foray *et al.* (2012). Protein content was assessed using Bradford's method (Bradford, 1976), using the Bio-Rad Protein Assay Kit II (Bio-Rad Laboratories, Inc., Copenhagen, Denmark), with bovine serum albumin serving as the standard. Total carbohydrate and glycogen contents were determined using anthrone reagent (CAS: 90448, Sigma-Aldrich, Stockholm, Sweden) prepared in 95% sulphuric acid, with D-glucose (1 mg mL^{-1}) as a standard (Van Handel, 1985). Lipid content was measured following a chloroform-methanol extraction step, using vanillin (CAS: 121335, Sigma-Aldrich), dissolved in 85% phosphoric acid, with olive oil (1 mL mL^{-1}) as a standard (Van Handel, 1985). The absorbance readings for total carbohydrates (carbohydrate and glycogen), lipids and protein analyses were measured in 96 well plates at 625 nm, 525 nm and 595 nm, respectively, using a Multiskan™ FC Microplate Photometer (Thermo Scientific™, Stockholm, Sweden). The concentration of carbohydrate, glycogen, lipid and protein was derived from standard curves, adjusted for the dilution, and normalised to the mean wing size of the mosquitoes from each treatment. For each treatment group, ten females were randomly selected and analysed.

4.4 Feeding assays

To assess how external stressors, elevation in CO_2 and extended egg quiescence period, affect the feeding behaviour of *Ae. aegypti*, no-choice feeding assays were conducted during the peak diel activity period of female mosquitoes at Zeitgeber time (ZT) 9-12 (Taylor and Jones, 1968), in the respective climate chambers. To assess the proportion of mosquitoes feeding and volume imbibed, females were given access to either honey or blood, while females with access to only water was used as controls for the volumetric analysis. After feeding, females were carefully placed into 1.5 mL Eppendorf tubes and immediately frozen at -20 °C until further analysis.

Honey feeding assays were performed on either teneral (Paper I and III) or five days post-emergence (Paper III) females, with 20-to-25 sugar-starved individuals per cage, for a duration of 3 h. The honey solution contained xylene cyanole (1 mg ml-1; FF; CAS 2650-17-1; Sigma-Aldrich), to facilitate scoring of engorged individuals and volumetric analysis of the imbibed meal. The calorimetric absorbance was measured at 620 nm in 96-well plates, with unfed females as controls and a standard curve generated with different volumes of honey (Paper I and III).

Blood feeding assays were performed on teneral females (Paper I), in which sugar-starved individuals were given access to sheep blood via an artificial membrane system for 1 h. The abdomens of fed and unfed females were dissected and volumetric analysis performed according to the haemoglobinometry method by Briegel *et al.* (1979). A standard curve was generated using different volumes of sheep blood (Paper I).

4.5 Transcriptome analysis of the peripheral olfactory system

The impact of two environmental stressors, including elevated CO_2 and extended egg quiescence duration, on transcriptional regulation of genes in the peripheral olfactory tissues was assessed through transcriptomic analysis. Antennal and maxillary palp tissues were collected from teneral females (30 ± 6 h) at ZT 10-12, prepared and stored in RNAlater (Thermo Fisher Scientific, Gothenburg, Sweden) until RNA extraction. A total of 16 antennal libraries were generated, with each library comprising pooled tissues from 50 individuals per replicate from different cohorts, across two CO_2 conditions, two egg quiescence periods, and four biological replicates 800 pairs of tissues. Similarly, 16 maxillary palp libraries were constructed using the same pooling strategy, yielding an additional 800 pairs of tissues. In total, 1,600 pairs of tissues were collected for all 32 libraries.

The total RNA extraction procedure was performed using the RNeasy microRNA kit (Qiagen, Hilden, Germany), following the manufacturer's protocol (Paper II) and stored at -80 °C. Later, the quality and concentration of total RNA were quantified using the TapeStation system 4150 (Agilent Technologies, Inc, Santa Clara, US) and RNA samples shipped for sequencing. The total RNA was used to create INVIEW ultra-low transcriptome libraries, using NovaSeq Illumina genome sequencing (Illumina NovaSeq 6000 S4 PE150 XP). The cDNA was constructed using the Eurofins proprietary protocol, generating 2 ×150 bp coverage paired-end reads with a depth of 20 million paired-end reads.

Prior to quantitative analyses on the library sequences, the raw reads underwent quality control steps involving the removal of adapter sequences and discarding sequences with a Phred score below 40, using CLC Genomics Workbench (23.0.5, Qiagen, Aarhus, Denmark). The cleaned sequences were mapped to the *Ae. aegypti* reference genome (VectorBase: *Aedes aegypti* LVP_AGWG, AaegL5.3). The transcript abundance is reported in transcripts per million (TPM), which accounts for differences in transcript length and sequencing depth across sample replicates.

To assess the effect of the two environmental stressors, CO_2 level and egg quiescent duration, on overall gene expression in the antennal and maxillary palp libraries, a principal component analysis (PCA) was performed. The high-dimensional dataset containing the antennal and maxillary palp libraries was projected onto two-dimensional components to determine the variance between libraries using the toolbox for RNA-seq data in CLC Genomics Workbench. Reliable expression of genes was determined at a threshold of 1 TPM. Differential expression of genes was analysed, generating a mean abundance value, fold change (FC) and false discovery rate (FDR). Genes were considered significantly differentially expressed when the $FC > 1.5$ and FDR $p\text{-value} < 0.05$.

Functional ontology analyses, including gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were performed on the differentially expressed genes across comparisons of CO_2 levels and egg quiescence duration in VectorBase (AaegL5.3, Release 68). The VectorBase GO enrichment tool was used for assessing the molecular function, with both computed and curated evidence limited to GO slim terms. The VectorBase metabolic pathway enrichment was used for KEGG analysis. The significance cut-off was set to alpha (α) = 0.05. Additionally, targeted effects induced by elevation in CO_2 and egg quiescence duration were investigated for select chemosensory gene families, including ORs, IRs, GRs, OBPs, CSPs and other non-canonical chemosensory gene families in the antennae and maxillary palps.

4.6 Olfactory-guided behavioural analysis of female mosquitoes

The behavioural response of females, of all genotypes, to synthetic floral odour blends was assessed in a two-choice Y-tube olfactometer (88 cm total length × 10 cm i.d.). The plexiglass Y-tube olfactometer was illuminated from above with white light (50 lx) (Fig. 1), and a charcoal-filtered humidified air stream (0.30 m s^{-1}; 25 ± 2 °C; and 65 ± 2% relative humidity) entered the olfactometer from the upwind side. A synthetic odour blend or solvent control (pentane) was delivered at the upwind end of the olfactometer to either arm via Teflon^{TM} tubing. The odour-delivery system consisted of a charcoal-filtered humidified air stream (1 L min-1), which was directed into two wash bottles (500 mL; Lenz Laborglas, Wertheim, Germany), each holding a wick dispenser (Karlsson *et al.*, 2017) made up of a cotton wick in a Teflon tube (30 mm long × 1.5 mm i.d.), inserted through a hole in a cap of a 1.5 mL glass vial, from which the synthetic floral odour blend or pentane was released. The wick dispenser allows the release of compounds at a constant rate, while maintaining the blend composition throughout the duration of the experiment (Karlsson *et al.*, 2017). An exhaust at the downwind end of the olfactometer removed excess odour.

Age- and mating-state-controlled female mosquitoes, across all genotypes were deprived of sucrose 24 h prior to the experiment, but had *ad libitum* access to distilled water. Individual females were then aspirated into release cages (8 cm × 9.3 cm i.d.) and allowed to acclimatise for 1 h. The release cage was inserted at the downwind end of the olfactometer, and females were allowed 2 min to acclimatise. The odour delivery tubes were then connected to each of the arms, and the release cage door was gently opened. Each mosquito was given 5 min to choose between the two arms, and mosquitoes that did not take off or make a choice were counted as "non-responders" and were not included in future analysis. To avoid bias, controls were performed at each experimental day, before the onset of the experiments, in which individual mosquitoes were allowed to choose between two arms without any stimuli. Furthermore, the synthetic blend and solvent control were exchanged between each replicate. All experiments were conducted during the peak activity of the mosquitoes, at ZT 9-12 (Taylor and Jones, 1968).

Two synthetic floral odour blends were prepared based on the composition of bioactive compounds identified through previous combined GC-EAD analysis of the headspace extract of *Lantana camara* and *Senna didymobotrya* (Wondwosen *et al.*, in prep). The Y-tube olfactometer experiments for females, of all genotypes, with the *L. camara* synthetic odour blend were performed to obtain a dose-response curve, and then an identified behaviourally active dose was used for subsequent experiments. Subtractive blend assays were designed to assess the importance of the natural ligand of the Or117 in eliciting the observed behavioural preference of unmated teneral females towards *L. camara*. The subtractive blends maintained the composition of the synthetic *L. camara* odour blend, with the same concentration of compounds, by making up the missing volume of camphor with pentane. The *L. camara* floral synthetic odour was used to assess the age-dependent behaviour in males towards floral odour.

5. Summary and discussion of results

5.1.1 Environmental stressors affect larval and adult life history traits of *Aedes aegypti* (Paper I)

Rising atmospheric CO_2 levels are a leading driver of anthropogenic climate change (Nunes, 2023), with concentrations predicted to increase exponentially up to 1000 ppm by the end of the century (IPCC, 2018). This increase is expected to be accompanied by shifts in rainfall patterns, water availability and relative humidity (Trenberth, 2011), which can profoundly influence various phenotypic responses and population dynamics of insects (Bujan *et al.*, 2016; Thorat and Nath, 2018). A crucial determinant of insect survival is their capacity to adapt to environmental changes through plasticity in life-history traits (Brass *et al.*, 2021). Such adaptive responses may involve alterations in developmental duration, starvation tolerance, adult size, metabolic reserves and feeding strategies, all of which contribute to maintaining population stability in fluctuating environments (Nylin and Gotthard, 1998). This study investigates the effects of two environmental stressors on *Ae. aegypti*, demonstrating that elevated CO_2 levels significantly impact both immature and adult life-history traits, an effect further modulated by the egg quiescence period.

The effect of elevation in CO_2 levels on larval developmental duration and larval survival was modulated by the extent of the egg quiescence period (Figure. 1). An extended egg desiccation period can exhaust maternally derived energy reserves supporting the pharate larvae, which increases the susceptibility of emerging larvae to suboptimal or stressful larval environments (Perez and Noriega, 2012). The delayed developmental duration and reduced survival observed in larvae that emerge from extended egg quiescence and are reared under elevated CO_2 are suggestive of such an energy limitation (Hahn and Denlinger, 2007). During larval development, attaining a critical energy reserve through active feeding is vital for pupation (Chambers and Klowden, 1990). However, the threshold reserve requirement is subject to change to adapt to stressful environments (Chambers and Klowden, 1990). The two adaptive developmental time shifts employed by larvae from two egg quiescence periods, when reared under elevated CO_2, indicate their cost-benefit strategy. Mosquito larvae display similar effects in response to various environmental stressors, such as temperature, photoperiod, resource limitation and larval density (Couret *et al.*, 2014; Couret and Benedict, 2014; Huxley *et al.*, 2021). A delay in developmental duration observed in larvae that emerged from older eggs essentially increases their food foraging time, but with a trade-off of increased risk of predation and habitat fluctuations (Clements, 1999). However, larvae emerging from newer eggs respond to an elevation in CO_2 level by a shortened developmental duration, likely a defence strategy metabolically affordable to larvae emerging from a short egg quiescence period.

To understand the mechanism of how elevation in CO_2 could affect aquatic life stages, an artificial increase in acidity was performed. Elevation in CO_2 levels has been shown to cause acidification in freshwater and marine ecosystems (Orr *et al.*, 2005; Hasler *et al.*, 2018), affecting the physiology of inhabiting organisms (Michaelidis *et al.* 2005; Abbey-Lambertz *et al.*, 2014; Jeffrey *et al.*, 2018) through either acidification of bodily fluids (Badre *et al.*, 2005) or hypercapnia (Michaelidis *et al.*, 2005). Artificial reduction in larval water pH, in the absence of CO_2, did not affect the life history traits of *Ae. aegypti* (Figure. 5), similar to studies performed on other aquatic organisms (Weiss *et al.*, 2018). This suggests that pH alone is not a stressor under elevated CO_2 and that an underlying, yet unidentified, mechanism is at play.

Environmental conditions experienced by larvae, including elevated CO_2 levels, influence the adult phenotype through carry-over effects on size, survival, energy reserves and feeding strategies (Takken *et al.*, 2013; Dickson *et al.*, 2017; Ezeakacha and Yee, 2019; Evans *et al.*, 2021). While elevated CO_2 levels during the aquatic stage differentially affected adult body size, the observed differential starvation tolerance is likely modulated by both carry-over effects and adult exposure during the starvation period, with an interaction with egg quiescent period (Figure. 2). One notable observation was the increased starvation tolerance in males and females emerging from extended egg quiescence periods, when reared under 1000 ppm CO_2 level, suggesting metabolic priming, *i.e.*, an anticipatory metabolic adjustment in adults as a result of stress experienced during larval stages (Perez and Noriega, 2012; Dittmer and Gabrieli, 2020). In adults emerging from either of the two egg quiescence durations, when reared under 1000 ppm CO_2 levels, body size did not significantly correlate with survival or energy reserves (Paper I), suggesting that metabolic priming is hampering the correlation between metabolic reserves and adult starvation tolerance.

Metabolic reserves in teneral adults are carryovers from larval reserve synthesis and largely determine the adult foraging decisions (See 2.3. Energy reserves and metabolism). Teneral females accumulated differential amounts of glycogen and lipids from larval stages, in response to the experienced egg quiescence period and CO_2 conditions (Figure. 3). The glycogen and lipid levels in teneral females emerging from different egg quiescence periods reveal distinct strategies of synthesis and utilisation of energy reserves in the transition from larval to adult stages in light of environmental stress. Overall, reduced carbohydrate and stored reserve levels in females in response to the two stress factors suggest low energy for flight, especially for foraging for their first meal (Nayar and Van Handel, 1971). However, no compensatory feeding on honey or blood was observed in relation to teneral reserves (Figure. 4), likely due to metabolic priming during the larval stage or a general low motivation to seek resources due to low energy.

Together, these findings provide strong evidence that environmental factors, such as elevation in CO_2 and desiccation duration, can interactively shape key life history traits of mosquitoes, with direct implications for population dynamics and vectorial capacity. Furthermore, adaptive responses, such as metabolic priming and altered feeding behaviours in response to environmental stressors, should be integrated into future predictive models to more accurately forecast vector ecology and disease transmission risk.

5.1.2 Impact of environmental stress on gene expression in peripheral olfactory system of *Aedes aegypti* (Paper II)

Predicted elevation in CO_2 and extended egg quiescence period affected adult life history traits and the feeding response of *Ae. aegypti* (Paper I). The differential feeding strategy employed by females in response to two environmental stressors highlights the behavioural plasticity, which has serious implications for disease transmission (Scott and Takken, 2012). Female feeding behaviour is the end process of the resource-seeking strategy, which is predominantly guided by olfactory cues. The resource-seeking behaviour of mosquitoes is modulated by external environmental factors (Majeed *et al*., 2014; Hagan *et al*., 2018; Vanderplanck *et al*., 2021;

Lahondere *et al*., 2023). Short or developmental exposure of insect species to abiotic stressors, including elevated CO_2, affect olfactory-guided behaviours (Stange, 1997; Sun and Ge, 2010; Majeed *et al*., 2014; Boullis *et al*., 2016; Boullis *et al*., 2017; Wang *et al*., 2023), sensory detection (Stange and Wong, 1993; Stange *et al*., 1995; Riveron *et al*., 2009; Martin *et al*., 2011; Majeed *et al*., 2014), as well as chemosensory gene expression in the peripheral olfactory system (Riveron *et al*., 2013; Guo *et al*., 2025; see also 2.6.2 Role of external environment).

A principal component analysis, followed by functional ontology enrichment analyses, revealed that both CO_2 level, and egg quiescence period modulated the expression of genes in the peripheral olfactory system of teneral female *Ae. aegypti* (Figure. 6). The high number of differentially expressed genes observed in response to elevated CO_2 level and egg quiescence period is indicative of the combined stress response in the olfactory tissues, especially the maxillary palp. The differentially expressed genes were primarily associated with energy metabolism, xenobiotic biodegradation and chemosensation. Notably, the influence of CO_2 level was gene family-specific, whereas the egg quiescence period had a distinct and overall effect, especially in the maxillary palp. While CO_2 level did not have a generalised overall effect on gene expression in the peripheral olfactory system, the differential feeding response displayed in response to the two stressors is likely mediated via the transcriptional regulation of specific gene families, as evident from the Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) analyses (Figure. 7 and 8). The transcriptional regulation of genes involved in energy metabolism and xenobiotic response pathways, identified through KEGG analysis, predominantly in the maxillary palp is indicative of the stress response in an organ mediating detection of CO_2 and other salient VOCs (Grant and O'Connell, 1995; Grant and Dickens, 2011; Vainer *et al*., 2024). Similar regulation of genes involved in metabolic pathways has been demonstrated in other insects, such as fruit flies and termites, under high CO_2 levels (Helenius *et al*., 2009; Wu *et al.,* 2016). The regulation of genes involved in energy metabolism is suggestive of high energy demands to adapt to the stressful environment (Arrese and Soulages, 2010). Elevation in CO_2 could manifest in insects as oxidative stress (Li *et al*., 2017) and/or acidification of bodily fluid, including the sensillum lymph, by the accumulation of carbonic acid (Brown *et al*., 1984). Such alteration of sensillum lymph can disturb the protein folding of OBPs, hindering their functional role (Zubkov *et al*., 2005; Manoharan *et al*., 2013; Mam *et al*., 2023). Such changes in the peri-receptor space may explain the observed transcriptional regulation of xenobiotic response genes, which also involve OBPs and ODEs (Lu *et al*., 2021; Abendroth *et al*., 2023). While mosquitoes can regulate ionic balance, how prolonged developmental exposure to elevated CO_2 can affect membrane-bound receptors and neuronal signalling (Chung *et al*., 2014; Porteus *et al*., 2018; Porteus *et al*., 2021) warrants further investigation.

Elevated CO_2 and extended egg quiescence duration differentially affect the gene transcript abundance of soluble and membrane-bound chemosensory genes, which potentially may alter the female resource-seeking behaviour (Figure 9). The observed transcriptional regulation of OBPs, particularly in the maxillary palp, is indicative of their role in elevated CO_2-induced stress response. However, of the differentially expressed *OBPs,* the functional role of *OBP22* and *OBP39* is currently known to be involved in detection of long-chain fatty acids in host- and oviposition-site-seeking, respectively (Leal and Leal, 2014, Wang *et al*., 2020). The notable regulation of several *OBPs* and *CSPs*, primarily in the maxillary palp, indicates an altered peri-receptor environment surrounding the OSNs, in response to the two stressors. Thus, under external stressors, the observed *OBP* and *CSP* regulation will likely affect the interaction between VOCs and membrane-bound receptors.

The observed differential regulation of membrane-bound receptors, including *ORs* in the antennae, in response to the two external stressors support the hypothesis that *OR* gene regulation mechanism is sensitive to early life environmental stressors (Jafari and Alenius, 2015). Although the functional implications of these transcriptional regulations remain largely unresolved, it is noteworthy that the majority of *OR* gene expression in both antennae and maxillary palps is unaffected. Acute exposure to elevated background CO_2 levels has been shown to affect the resource-seeking behaviour in mosquitoes through sensory constraints (Majeed *et al.,* 2014). However, developmental exposure to high CO_2 levels does not modulate the expression of CO_2 receptor genes, Gr1, Gr2 and Gr3 (Erdelyan *et al*., 2012; McMeniman *et al*., 2014). This suggests that behavioural or electrophysiological changes, similar to those observed during short-term elevated CO_2 exposure (Majeed *et al*., 2014) may underlie the effects of long-term exposure.

This study demonstrates that predicted climate change factors, particularly elevated atmospheric CO_2 interact with other stressors, such as prolonged egg quiescence period, to modulate gene expression and elicit a stress response in the peripheral olfactory system of female *Ae. aegypti*. These molecular changes may impair the female mosquito's ability to detect ecologically-relevant VOCs, potentially disrupting critical resource-seeking behaviours. While prior studies have shown that increased CO_2 levels and extended egg desiccation duration negatively affect life-history traits across aquatic and terrestrial stages of *Ae. aegypti,* with documented carry-over effects on adult feeding strategies, their combined impact on olfactory function at the electrophysiological and behavioural level warrants further investigation.

5.1.3 Odorant receptor *Or117* regulates female floral-seeking (Paper III)

As mentioned above, female *Ae. aegypti* display a wide repertoire of odour-mediated behaviours that are temporally dynamic and correlated with the regulation of chemosensory genes, including *ORs*, in the peripheral olfactory system (See 2.5.3. Internal state dependent plasticity). While there is a general concerted increase in chemosensory gene transcript abundance with age, the ones not following this regulated pattern have been proposed to function as molecular switches, modulating the transition from one behavioural state to another (Hill and Ignell, 2021). The differential modulation of chemosensory receptors, including *ORs* and *IRs*, which are involved in regulating attraction and discrimination, may lead to altered perception of odours through altered combinatorial receptor codes. In *Ae. aegypti*, the age-dependent behavioural plasticity displayed by females towards floral sources coincides with the downregulation of *Or117*, one of the two Ors defying the concerted upregulation of chemosensory gene expression pattern (Tallon *et al.,* 2019; Hill and Ignell, 2021).

The functional characterisation of *Or117*, by heterologous expression in the empty neuron system of *Drosophila,* identified 1*R*-(+)-camphor and 1*S*-(-)-camphor, constituent of *Lantana camara* floral scent, one of the most-preferred nectar sources of *Ae. aegypti* (Wondwosen *et al.,* in prep), as eliciting the highest excitatory response. This is followed by terpenoids, including (+/-)-α-terpineol, (+)-limonene oxide, α-copaene, and benzaldehyde, as additional ligands, which form major chemical classes constituting a floral odour (Knudsen and Gershenzon, 2006). The decline in female reliance on floral sources for metabolic needs with age, correlated with the regulation of *Or117*, and its functional role, poses this receptor as a potential molecular switch regulating floral-seeking in teneral females.

To investigate the mechanism underlying this regulation, a targeted null mutation in *Ae. aegypti Or117* was performed using the CRISPR/Cas9 gene editing system (Figure 10). In a Y-tube olfactometer, wildtype females displayed an age- and mating-state-dependent response towards *L. camara* synthetic floral odour blend. Furthermore, this effect was reflected in the differential volume of honey imbibed by females in response to their internal physiology and energy demands (Foster, 1995). The targeted knockout of *Or117* abolished the attraction of teneral females towards the synthetic floral *L. camara* odour, while feeding behaviour of homozygous mutants did not differ from their wildtype counterparts. To further assess the role of Or117 and its ligand, camphor, the response of wildtype females was tested to a subtractive *L. camara* synthetic floral odour blend, deprived of camphor, demonstrating the requirement of the ligand in mediating floral seeking. The observed abolishment of attraction in unmated teneral homozygous females establishes a causal relationship between the chemosensory receptor gene regulation and ensuing floral-seeking in *Ae. aegypti* females, similar to the causal relationships established in regulating odour-guided behaviours, including host seeking in *Drosophila* (Auer *et al.*, 2020) and repellence in *Ae. aegypti* (Liu *et al.*, 2021), respectively.

The effect of ageing, mating and mutation of Or117 on the detection of *L. camara* floral VOCs was assessed through electrophysiological recordings. Wildtype females displayed differential age- and mating-dependent responses to the *L. camara* synthetic floral odour blend, to which there was an off-and-on response to six out of ten *L. camara* floral VOCs as a function of the internal physiology of the female. However, the sensitivity of antennal OSN to the rest of the four compounds, including camphor, did not alter in response to age or mating (Paper III).

The knockout of *Or117* rendered females anosmic to camphor across all ages and mating states, emphasising the requirement of this receptor for the antennal detection of this floral VOC. Notably, the knockout of *Or117* also affected the detection of β-ocimene and 4-terpineol in an age- and mating-dependent manner, indicating a plausible feedback mechanism mediated by *Or117* affecting gene stability and expression of co-expressed Ors in the OSN (Maguire *et al.,* 2022). Single-nucleus RNA transcriptome analysis reveals that the Or117-expressing OSN co-expresses two other Ors, Or116 and Or130 (Adavi *et al.,* 2024). However, whether these receptors are tuned to β-ocimene and 4-terpineol (Chen *et al*., 2025) remains to be investigated. The observed age- and mating-dependent response to two above-mentioned VOCs in homozygous mutants suggests that their cognate receptor expression is modulated in response to internal physiological states, which demands further investigation.

This study demonstrates that female *Ae. aegypti* display age- and mating-dependent floral-seeking behaviour in relation to their energy and reproductive demands. The odorant receptor, *Or117* and its ligand, camphor, is required for the attraction of teneral females towards a synthetic *L. camara* floral odour blend, which demonstrates a direct causal molecular switch regulating resource-seeking behaviour. Such regulation in the peripheral olfactory system to accommodate dynamic behavioural state changes is likely an evolutionarily energy-efficient mechanism (Bruce and Pickett, 2011; Hill and Ignell, 2021).

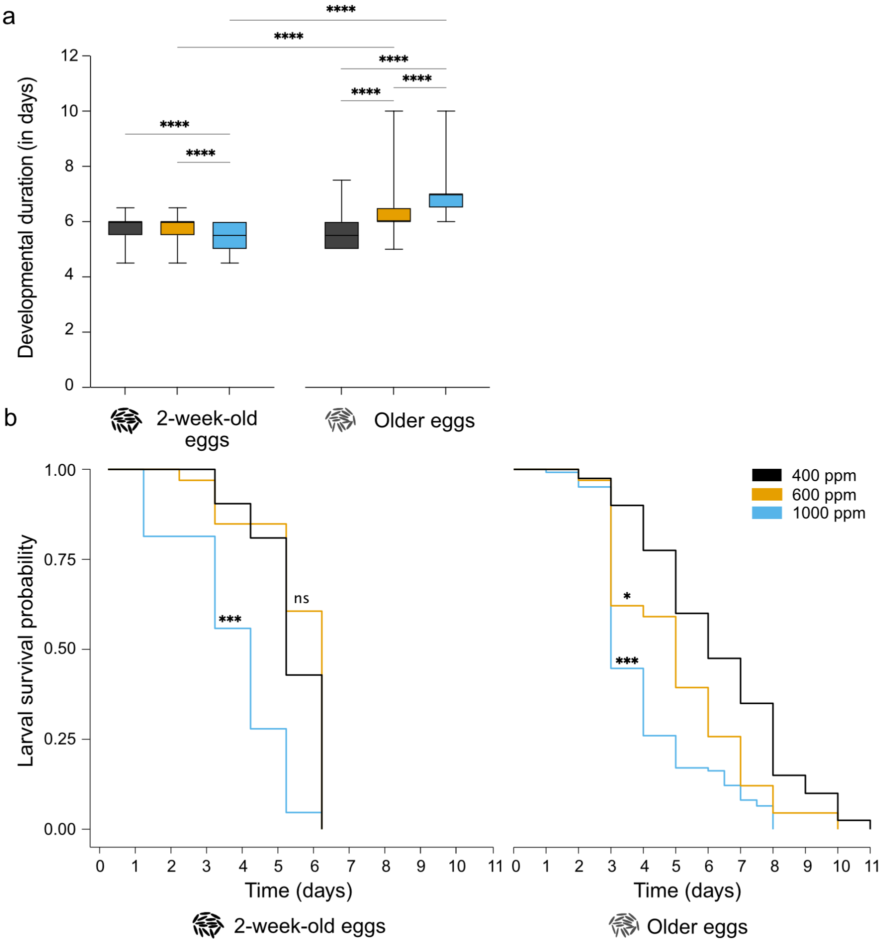

Figure 1. The effect of extended egg quiescence and elevated CO_2 level on immature stage development and survival.

(a) Developmental duration of the immature stage from larvae to pupae. For comparison between groups, a Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed. The whiskers denote minimum to maximum, and asterisks indicate significant differences between the groups ($N = 3$, $n = 300$ larvae, $p < 0.05$). (b) Survival probability of the larvae originating from 2-week- and 3-6 months-old (older) eggs. The curves were analysed using a Cox regression model, followed by a log-rank *post-hoc* test using the 'survival' package ($N = 3$, $n = 300$ larvae, $p < 0.05$).

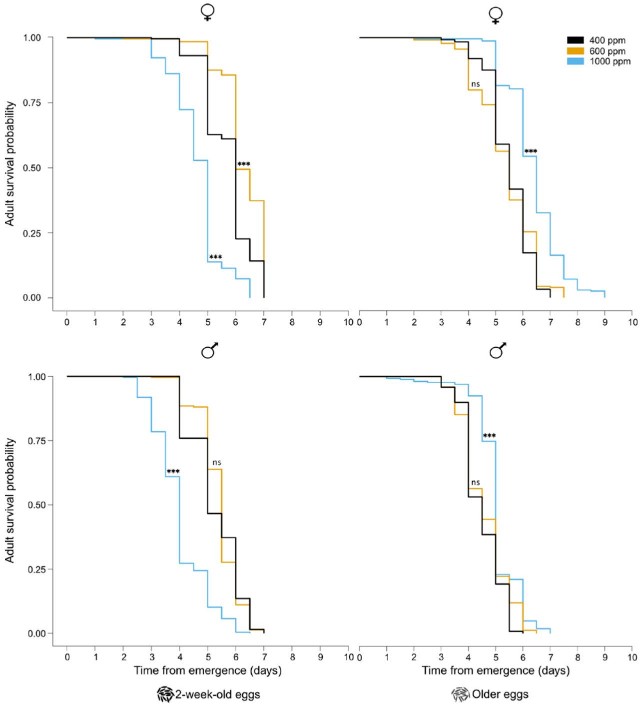

Figure 2. The interactive effect of extended quiescence and elevation in CO_2 level on the survival of adult *Aedes aegypti.*

Survival probability curves of the adults are separated by sex and egg quiescence period. The curves were analysed using a mixed-effects Cox regression model, followed by a log-rank post-hoc test using the ‘survival’ package (n (per group) = 245-250, $p < 0.05$).

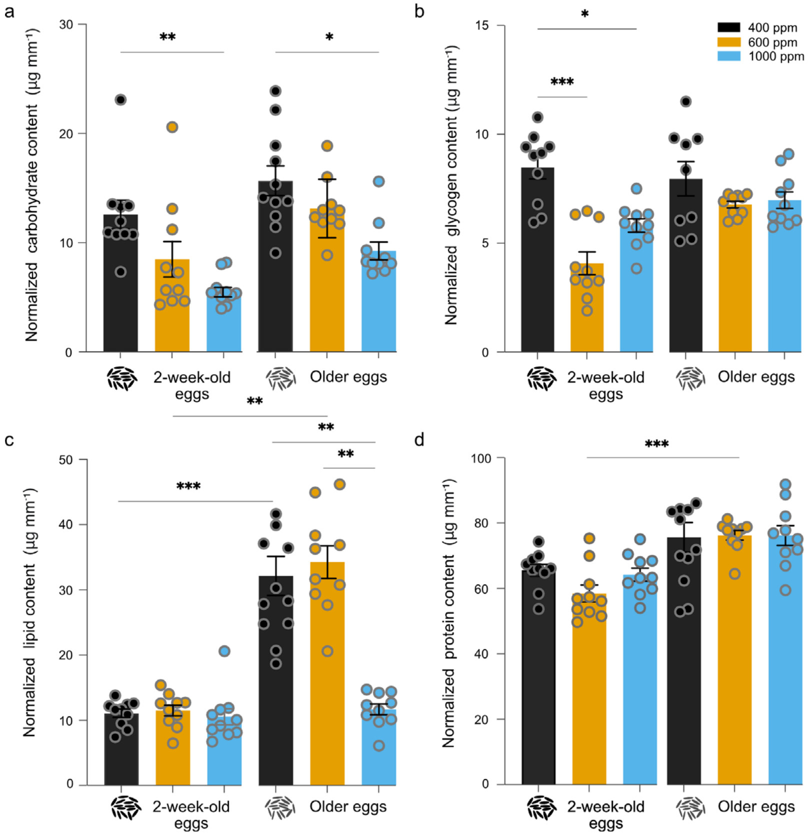

Figure 3. Metabolic reserves accumulated by teneral females in response to elevated CO_2 levels and egg quiescence.

The amount of soluble (a) carbohydrate, (b) glycogen, (c) lipid and (d) protein content normalised for body size. The error bars represent the standard error of the mean, and asterisks denote significant differences between the groups. For comparison between groups, a Kruskal-Wallis test followed by Dunn’s multiple comparisons test was performed ($n = 10$, $p < 0.05$).

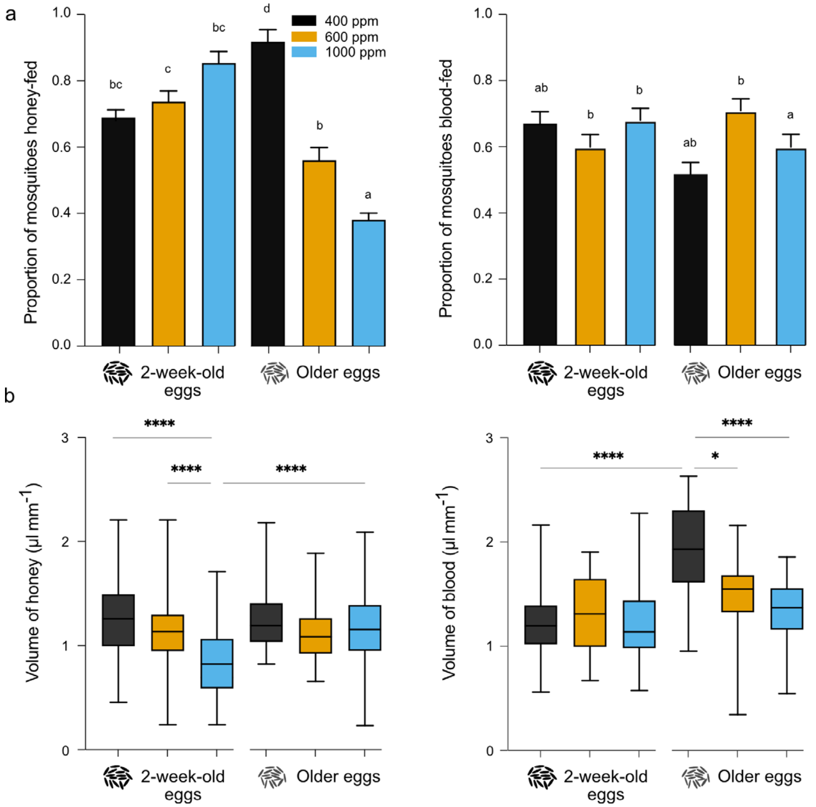

Figure 4. Differential feeding of female *Aedes aegypti* in response to elevated CO_2 conditions and extended egg quiescence.

(a) The proportion of teneral females that fed on honey (left) and blood (right) was differentially and significantly affected by extended egg quiescence and elevation in CO_2 level. The bars represent mean (± SE) of proportion of females feeding and letters denote significant differences in pairwise comparisons using 'emmeans' Tukey method (n = 160-170 females, $p < 0.05$). (b) Volumetric analysis of imbibed honey (left) and blood (right) normalised for body size. For comparison between groups, a Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed. The whiskers denote the minimum to maximum values and asterisks indicate significant differences between the groups (n = 50 females, $p < 0.05$).

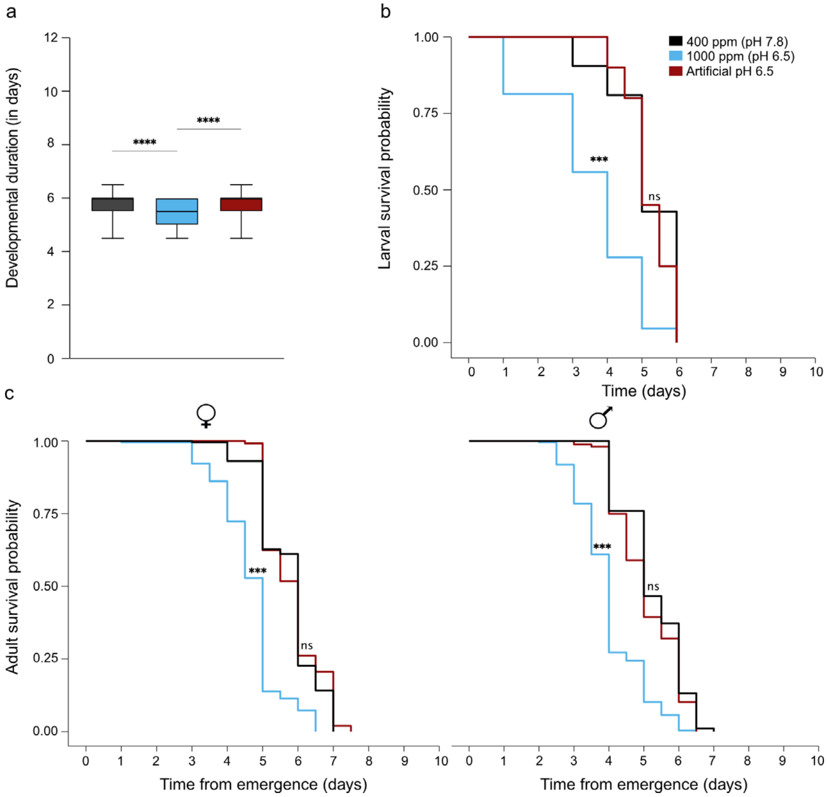

Figure 5. The effect of water acidification on the life history parameters of immature and adult stages of *Aedes aegypti*.

(a) Developmental duration of the immature stage from larvae to pupae. For comparison between groups, a Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed. The whiskers denote minimum to maximum values, and asterisks indicate significant differences between the groups ($N = 3$, $n = 300$ larvae, $p < 0.05$). (b) Survival probability of the larvae originating from 2-week-old eggs reared under ambient and 1000 ppm CO_2 conditions, as well as at pH 6.5. (c) Adult survival probability curves are separated by sex. The curves were analysed using a mixed-effect Cox regression model, followed by a log-rank *post-hoc* test using the 'survival' package ($n = 245$-250, $p < 0.05$). The data shown for ambient (pH 7.8) and 1000 ppm (pH 6.5) CO_2 are the same as in Figures 1 and 2.

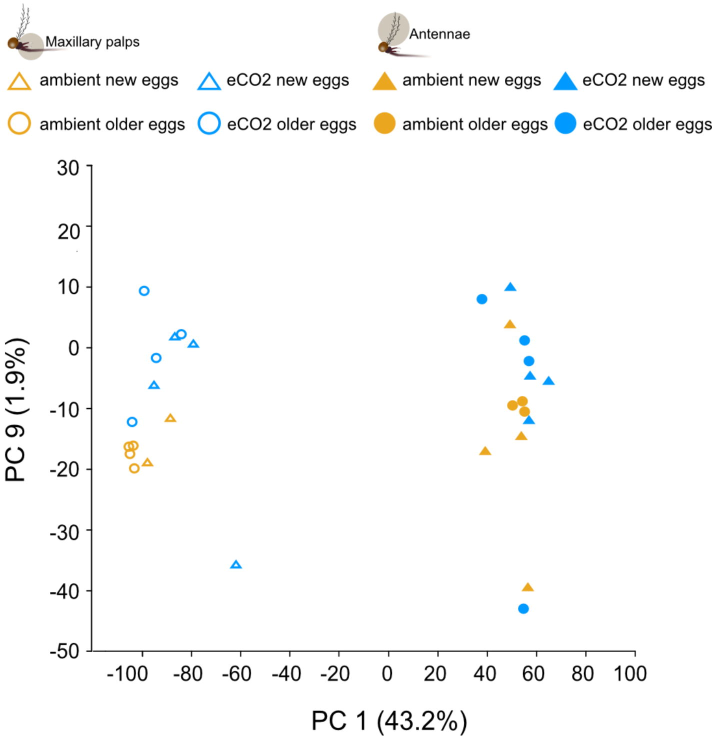

Figure 6. Overall effect of elevated CO_2, and egg quiescence, on gene expression in the peripheral olfactory organs of *Aedes aegypti*.

Principal component analysis of antennal and maxillary palp libraries of females emerging from new and older eggs, with short and extended egg quiescent duration, respectively, reared under ambient and elevated CO_2 conditions. A total of 29 libraries were analysed to estimate the change in overall gene expression, in which Principal Component (PC) 1 (43.2%) and PC 9 (1.9%) accounted for the variance between the libraries.

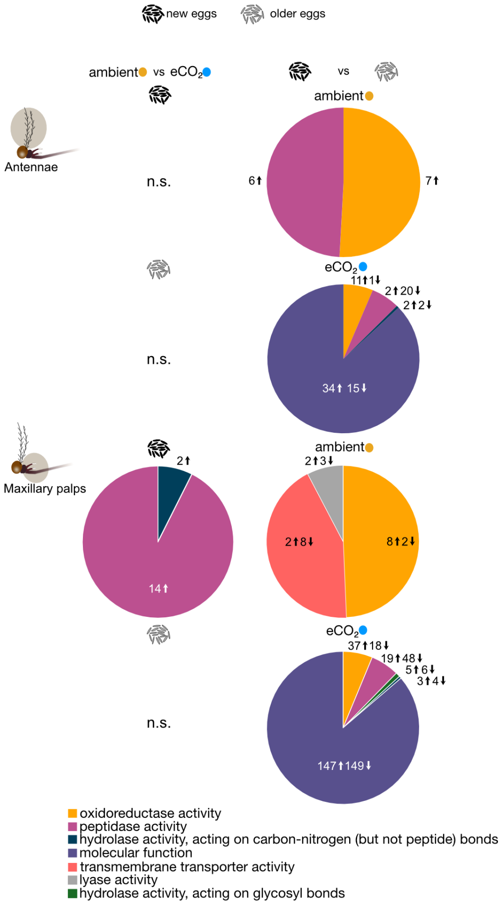

Figure 7. Gene ontology analysis of differentially expressed genes in the antennae and maxillary palps of *Aedes aegypti.*

The olfactory tissues were collected from females reared under ambient and extreme CO_2 conditions, as well as short and extended egg quiescence duration, referred to as new and older eggs, respectively. Pairwise comparisons are arranged in a matrix in response to CO_2 conditions and egg quiescence period. The differentially expressed genes are classified into molecular function ontology, using gene ontology slim categorisation. n.s.: non-significant.

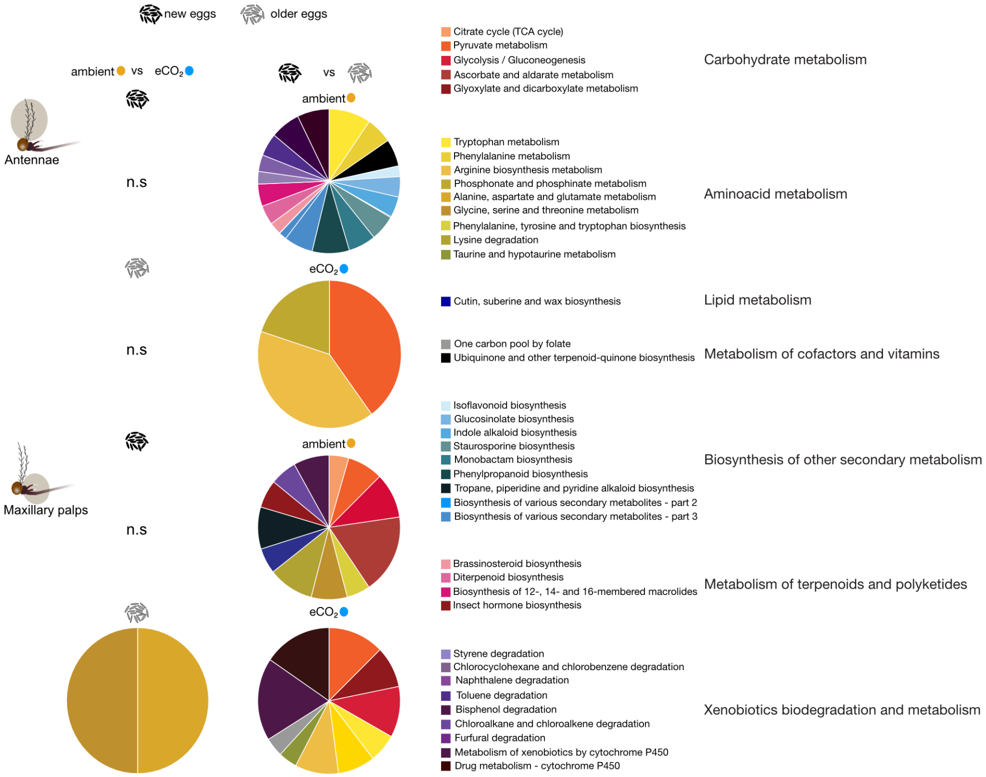

Figure 8. Kyoto Encyclopedia of Genes and Genomes pathway analysis of differentially expressed genes in the antennae and maxillary palps of *Aedes aegypti*.

The olfactory tissues were collected from females reared under ambient and extreme CO_2 conditions, as well as short and extended egg quiescence duration, referred to as new and older eggs, respectively. Pairwise comparisons are arranged in a matrix in relation to the response to CO_2 conditions (eCO_2) and egg quiescence period. The categories are annotated from Vectorbase and further classified into pathways designated by Kyoto Encyclopedia of Genes and Genomes database (https://www.genome.jp/kegg/).

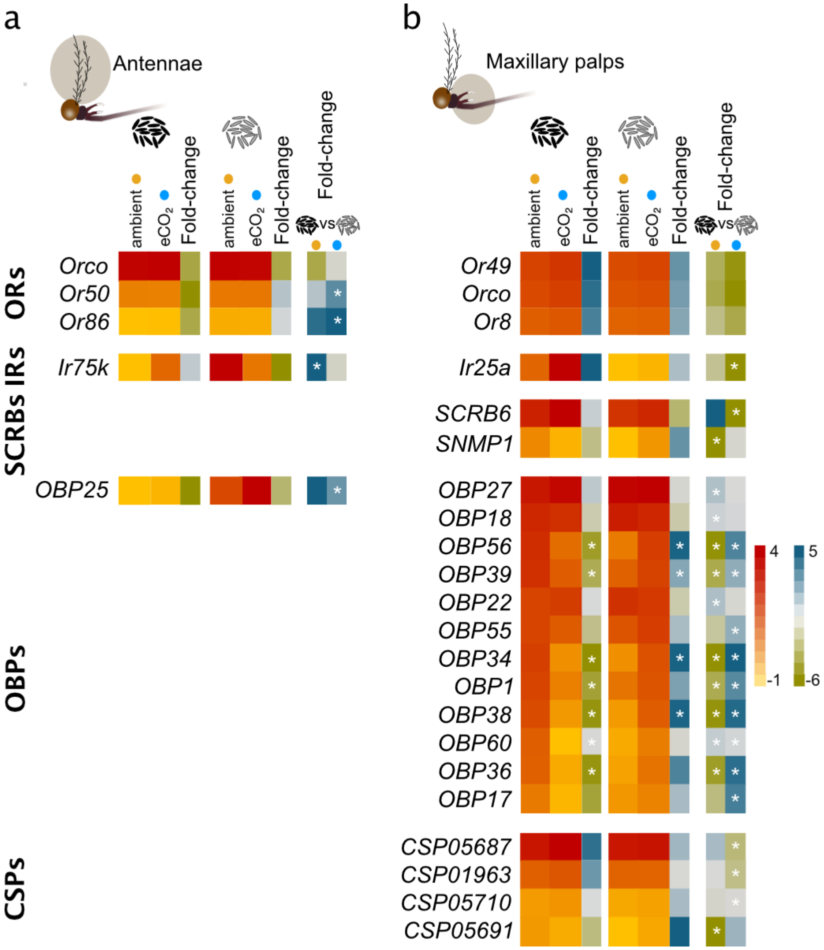

Figure 9. Differential abundance of chemosensory genes in *Aedes aegypti* in response to extreme CO_2 conditions and extended egg quiescence period

The olfactory tissues were collected from females reared under ambient and extreme CO_2 conditions, as well as short and extended egg quiescence duration, referred to as new and older eggs, respectively. The abundance of reliably expressed (>1 transcript per million) chemosensory genes was compared between ambient and elevated CO_2 (eCO_2) levels, as well as egg quiescent periods, in antennal (a) and maxillary palp (b) libraries, and demonstrated by fold-change (> 1.5-fold change; FDR > 0.05). Asterisks on fold change denote significant differences between pairwise comparisons.

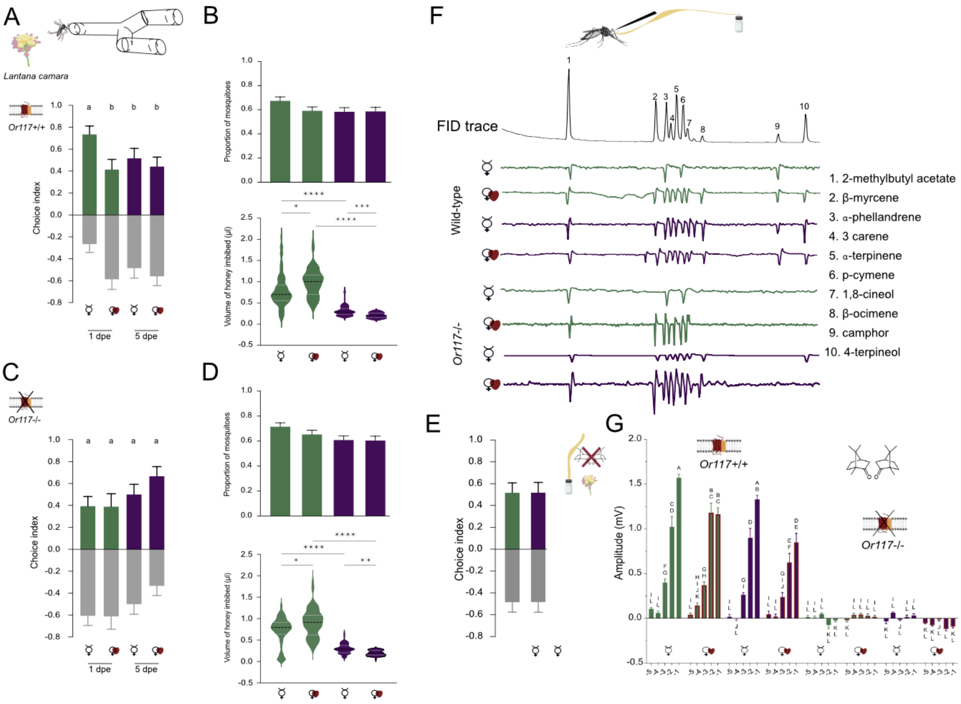

Figure 10. *Or117* regulates the floral seeking in *Aedes aegypti*

(A and C) Females display an age- and mating-state-dependent behaviour towards a synthetic floral blend of *L. camara*, in which Or117 is necessary for the behavioural attraction of teneral females. Behavioural response of age and mating-state controlled, wild-type and mutant females towards a synthetic floral blend of *L. camara* assessed in a Y-tube olfactometer. The bars represent choice index (± SE) and different letters denote significant differences between the genotypes (pairwise comparison using 'emmeans,' corrected with the Tukey method). ($n = 30$, $p < 0.05$). (B and D). Differential feeding of wild-type and mutant females as an effect of age and mating status. The proportion of females that fed on honey displays no effect of age or mating (above). The bars represent mean (± SE) proportion of females feeding and letters denote significant differences in pairwise comparisons using 'emmeans' Tukey method ($n = 200$ females, $p < 0.05$). Volumetric analysis demonstrated differential effect of age and mating on volume imbibed by females (below). For comparison of volume imbibed by females, a Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed. The asterisks indicate significant differences between the groups (n = 80 females, $p < 0.05$). (E) Camphor is necessary for the attraction of unmated teneral wild-type females towards *L. camara* synthetic floral odour source. Behavioural response of wildtype unmated teneral and older females towards the subtractive synthetic blend of *L. camara*. (F) Combined gas chromatography and electroantennographic detection (GC-EAD) analyses of wild-type (top) and mutant (bottom) mosquitoes demonstrate an age- and mating-state-dependent response towards a synthetic floral blend of *L. camara*. The FID trace is depicted above and sample trace from a single mosquito belonging to each physiological state and strain is depicted below (G). Electroantennographic analysis of age- and mating-state-controlled wildtype and mutant females demonstrates that mutant females are anosmic to camphor and no significant effect of age or mating on the sensitivity of females towards ecologically relevant doses of camphor (n=10 per group). The bars represent mean amplitude (± SE) of neuronal response and letters denote significant differences in pairwise comparisons Tukey's method.

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Popular science summary

Mosquitoes, the tiny, deadly hunters, have shaped human history through the widespread impact of mosquito-borne diseases. Their remarkable success as disease vectors has made them a critical focus of global public health research. Today, human-driven climate change is rapidly altering the environments mosquitoes inhabit, through rising temperatures, elevated CO_2 levels, shifting rainfall patterns, and changes in water biochemistry. These environmental shifts can profoundly influence multiple aspects of mosquito biology. In addition to external factors, internal physiological changes such as ageing and mating also modulate the behavioural repertoire displayed by female mosquitoes. This thesis investigates how both external environmental cues and internal physiological states shape mosquito biology, using approaches that span ecology, molecular biology, and neuroethology.

Under natural conditions, anthropogenic climate change introduces a complex array of interacting variables that affect both the larval and adult stages of mosquitoes. Among these, elevated atmospheric CO_2 levels and prolonged mosquito egg desiccation periods, resulting from unpredictable water availability, have been shown to interactively and negatively affect survival across both aquatic and terrestrial life stages. These environmental pressures not only regulate how energy reserves are synthesised and utilised, but also influence feeding strategies in female mosquitoes. Collectively, these changes have serious implications for mosquito population dynamics and the future spread of mosquito-borne diseases.

Female *Aedes aegypti* mosquitoes rely on their highly developed olfactory system to locate essential resources, including plants for sugar meals, human hosts for blood feeding, and suitable sites for egg-laying. Their resource-seeking behaviour dynamically shifts between plants and humans depending on internal physiological states and energy demands, potentially influenced by external environmental factors. However, the extent to which external stressors affect mosquito sensory systems remains poorly understood.

Evidence from other organisms, including insects, suggests that elevated CO_2 levels can impair olfactory-guided behaviours and alter the functioning of sensory neurons. To investigate this in mosquitoes, the effects of elevated

CO_2 levels and extended egg dormancy were assessed via transcriptome analyses on female olfactory tissues, the antennae and maxillary palp. These analyses revealed that exposure to environmental stressors resulted in significant changes in gene expression, most prominently within the CO_2-sensitive olfactory organ. Differentially expressed genes were associated with energy metabolism, xenobiotic detoxification, and olfactory processes. These findings point towards stress-induced affects at the level of gene regulation, with potential consequences for the olfactory capabilities and resource-finding efficiency of female mosquitoes.

From the resource-seeking perspective, the sugar-seeking behaviour in female mosquitoes has received less attention compared to blood-seeking behaviour. As noted earlier, the dynamics of sugar- and blood-seeking are closely linked to the internal physiological state of the female. Newly emerged adults primarily seek a plant sugar to meet the high energy demands required immediately after emergence. As females age, there is a gradual transition towards blood-seeking behaviour necessary for egg development. Correspondingly, there is a concerted upregulation of olfactory gene expression in the antennae and maxillary palps to support this behavioural shift. Interestingly, one olfactory receptor gene, *Or117,* defies this general trend. The expression of *Or117* gene decreases with female age. Functional characterisation of *Or117* through heterologous expression systems revealed that its primary ligand is a floral volatile found in *Lantana camara*, a highly preferred nectar source for female *Ae. aegypti*. We show that females display an age- and mating-dependent floral seeking. Using mutagenesis, behavioural assays, and electrophysiological analyses, we demonstrated that *Or117* plays a critical role in modulating age-dependent floral-seeking behaviour. Specifically, the odorant receptor, Or117 and its ligand are required for the attraction of unmated newly emerged females. The study provides a direct causal link between changes in the peripheral olfactory system and the flexible resource-seeking behaviour in female mosquitoes. Such plasticity in the olfactory system offers mosquitoes the ability to rapidly or gradually adjust their behaviours in response to internal physiological changes or external environmental pressures, a flexibility that likely contributes to their remarkable ecological success.

Populärvetenskaplig sammanfattning

Myggor, dessa små men dödliga jägare, har format människans historia genom en omfattande påverkan av myggburna sjukdomar. Deras anmärkningsvärda framgång som sjukdomsvektorer har gjort dem till ett centralt fokus inom global folkhälsoforskning. Idag förändrar människodriven klimatförändringar snabbt de miljöer som myggor bebor, genom stigande temperaturer, förhöjda CO_2-nivåer, förändrade nederbördsmönster och förändringar i vattenkemi. Dessa miljöförändringar kan i hög grad påverka flera aspekter av myggans biologi. Utöver yttre faktorer moduleras även honmyggans beteenderepertoar av interna fysiologiska förändringar som åldrande och parning. Denna avhandling undersöker hur både yttre miljösignaler och interna fysiologiska tillstånd formar myggans biologi, med hjälp av metoder som spänner över ekologi, molekylärbiologi och neuroetologi.

Under naturliga förhållanden introducerar klimatförändringar en komplex uppsättning av interagerande variabler som påverkar både larver och vuxna myggor. Bland dessa har förhöjda atmosfäriska CO_2-nivåer och förlängda uttorkningsperioder hos ägg, som uppstår till följd av oförutsägbar tillgång på vatten, visat sig interagera och negativt påverka överlevnaden av både akvatiska och terrestra livsstadier. Dessa miljörelaterade stressfaktorer styr inte bara hur energireserver syntetiseras och används, utan påverkar även födostrategier hos honmyggor. Sammanlagt kan dessa förändringar ha allvarliga konsekvenser på myggpopulationernas dynamik och den framtida spridningen av myggburna sjukdomar.

Honmyggor av *Aedes* aegypti är beroende av ett högt utvecklat luktsinne för att lokalisera viktiga resurser, inklusive växter för sockerintag, mänskliga värdar för blodmål och lämpliga platser för äggläggning. Deras resursökande beteende växlar dynamiskt mellan växter och människor beroende på interna fysiologiska tillstånd och energibehov, vilket potentiellt kan påverkas av yttre miljöfaktorer. Däremot är det fortfarande dåligt förstått i vilken utsträckning externa stressfaktorer påverkar myggans sensoriska system.

Evidens från andra organismer, inklusive insekter, tyder på att förhöjda CO_2-nivåer kan försämra luktstyrda beteenden och förändra sinnescellers funktion. För att undersöka detta i myggor analyserades effekterna av förhöjd

CO_2 och förlängd äggdvala genom transkriptomanalyser av det perifera doftsystemet, det vill säga antennerna och maxillarpalperna. Denna analys visade att exponering för miljöstressfaktorer leder till signifikanta förändringar i genuttryck, särskilt inom det CO_2-känsliga luktorganet. De differentiellt uttryckta generna var associerade med energimetabolism, avgiftning av xenobiotiska substanser och processer involverade i dofttigenkänning. Dessa fynd tyder på en tydlig stressinducerad effekt på genregleringsnivå, med potentiella konsekvenser för honornas kapacitet att detektera dofter och effektivt lokalisera resurser.

Från ett resurssöksperspektiv har honmyggors sockersökande fått mindre uppmärksamhet än deras blodletande. Som tidigare nämnts är dynamiken mellan socker- och blodletande nära kopplat till honans interna fysiologiska tillstånd. Nykläckta vuxna söker främst växtsocker för att möta de höga energibehov som krävs omedelbart efter kläckning. I takt med att honan åldras sker en gradvis övergång till blodletande, som är nödvändigt för äggutveckling. Denna förändring i beteende åtföljs av en samordnad uppreglering av gener som kodar för protein som binder in olika doftämnen i antennerna och maxillarpalperna. Intressant nog avviker en lav dessa gener, Or117, från detta generella mönster. Uttrycket av Or117 minskar med åldern. Funktionell karakterisering av Or117 genom ett så kallat heterologt uttryckssystem visade att dess primära ligand är en blomdoft som finns i *Lantana camara*, en växt som föredras av *Ae. aegypti* honor. Vi visar att honor uppvisar ett sockersöksbeteende som är beroende av ålder och parningsstatus. Genom mutagenes, beteendeanalyser och elektrofysiologiska tester visar vi att Or117 spelar en avgörande roll i att reglera detta åldersberoende beteendet. Specifikt krävs doftreceptorn Or117 och dess ligand för att attrahera oparade, nykläckta honor. Studien etablerar en direkt kausal koppling mellan förändringar i det perifera luktsystemet och den flexibla resursökande beteenderepertoaren hos honmyggor. Denna plasticitet i doftsystemet ger myggor en möjlighet att snabbt eller gradvis anpassa sina beteenden beroende av interna fysiologiska förändringar eller yttre miljöförändringar—en flexibilitet som sannolikt bidrar till deras anmärkningsvärda ekologiska framgång.

പ്രബന്ധസംഗ്രഹം

കൊതുകുകൾ പകരുന്ന രോഗങ്ങൾ മനുഷ്യ സമൂഹത്തെ വളരെ പ്രതികൂലമായി ബാധിച്ചിട്ടുണ്ട്. രോഗ വാഹകർ എന്ന നിലയിൽ അവയുടെ ആഘാതം ആഗോള പൊതുജനാരോഗ്യ ഗവേഷണത്തിന്റെ ശ്രദ്ധ പിടിച്ചുപറ്റിയിരിക്കുന്നു. എന്നിരിക്കേ, മനുഷ്യർ സൃഷ്ടിച്ച കാലാവസ്ഥാ വ്യതിയാനം, ഉയർന്ന താപനില, വർദ്ധിച്ച കാർബൺ ഡൈ ഓക്സൈഡ് (CO_2) അളവ്, മഴയുടെ രീതിയിലുള്ള മാറ്റങ്ങൾ, ജലത്തിന്റെ രാസഗുണത്തിലെ മാറ്റങ്ങൾ എന്നിവ കൊതുകുകൾ ജീവിക്കുന്ന സാഹചര്യങ്ങളെയും, അവയുടെ ജീവശാസ്ത്രത്തിന്റെ വിവിധ വശങ്ങളെയും സ്വാധിനിക്കുന്നു. ബാഹ്യ ഘടകങ്ങൾക്ക് പുറമേ, പ്രായം, ഇണചേരൽ തുടങ്ങിയ ആന്തരിക ശാരീരിക മാറ്റങ്ങളും പെൺ കൊതുകുകളുടെ പെരുമാറ്റങ്ങളെ നിയന്ത്രിക്കുന്നു. പരിസ്ഥിതി, തന്മാത്രാ ജീവശാസ്ത്രം, ന്യൂറോഎത്തോളജി തുടങ്ങിയ വിവിധ സമീപനങ്ങളിലൂടെ ഈ പഠനം ബാഹ്യവും ആന്തരികവുമായ ഘടകങ്ങൾ കൊതുകുകളുടെ ജീവശാസ്ത്രത്തെ എങ്ങനെ സ്വാധീനിക്കുന്നു എന്ന് പരിശോധിക്കുന്നു.

കാലാവസ്ഥാ വ്യതിയാനം കാരണം ഉയർന്ന CO_2 നിലകളും, വെള്ളം ലഭിക്കാതെ വരണ്ട സാഹചര്യങ്ങളിൽ മുട്ടകൾക്കുണ്ടാകുന്ന ദീർഘകാല പ്രശ്നങ്ങളും കൊതുകുകളുടെ ജീവൻ നിലനിർത്താനുള്ള കഴിവിനെ പ്രതികൂലമായി ബാധിക്കുന്നു. ഈ സാഹചര്യങ്ങൾ കൊതുകുകളുടെ ഊർജ്ജ സംഭരണവും, ഭക്ഷണ രീതികളും നിയന്ത്രിക്കുന്നു. ഇത് കൊതുകുകളുടെ എണ്ണം വർദ്ധിപ്പിക്കുന്നതിലും രോഗങ്ങൾ വ്യാപിക്കുന്നതിലും ഗൗരവമായ സ്വാധീനം ചെലുത്തുന്നു.

പെൺ കൊതുകുകൾ അവയുടെ ആന്തരിക ശാരീരിക അവസ്ഥകളും ഊർജ ആവശ്യകതകളും അനുസരിച്ച് ആവശ്യമായ വിഭവങ്ങൾ കണ്ടെത്തുന്നു. ഉദാഹരണത്തിന്, പുതുതായി ഉയർന്നുവന്ന പെൺ കൊതുകുകൾക്ക് ഊർജം ലഭിക്കാനായി സസ്യങ്ങളിൽ നിന്ന് തേൻ കുടിക്കണം, എന്നാൽ പ്രായമായാൽ മുട്ടകൾ വികസിപ്പിക്കാനായി മനുഷ്യരിൽ നിന്ന് രക്തം കുടിക്കാൻ അവയുടെ പെരുമാറ്റം മാറുന്നു. ഈ പെരുമാറ്റ വ്യതിയാനങ്ങൾ ബാഹ്യ പരിസ്ഥിതി ഘടകങ്ങളാലും സ്വാധീനിക്കപ്പെടാറുണ്ട്. എന്നാൽ, ഇത്തരത്തിലുള്ള ബാഹ്യ സമ്മർദ്ദങ്ങൾ കൊതുകുകളുടെ ഇന്ദ്രിയ സംവിധാനങ്ങളെ എത്രത്തോളം ബാധിക്കുന്നു എന്ന് ഇപ്പോഴും വ്യക്തമല്ല.

ഈ പഠനത്തിൽ, ഉയർന്ന CO_2 നിലകളും മുട്ടകൾക്ക് നേരിടുന്ന വരണ്ട സാഹചര്യങ്ങളും കൊതുകുകളുടെ ഗന്ധജ്ഞാന അവയവങ്ങളെ (ആന്റിനയും മാക്സില്ലറി പാൽപ്പ്) എങ്ങനെ ബാധിക്കുന്നു എന്ന് ട്രാൻസ്ക്രിപ്റ്റോം വിശകലനത്തിലൂടെ പരിശോധിച്ചു. ഇതിലൂടെ കണ്ടെത്തിയത്, ഈ സമ്മർദ്ദങ്ങൾ കൊണ്ട് ഗന്ധജ്ഞാനവുമായി ബന്ധപ്പെട്ട ജീനുകളുടെ പ്രവർത്തനം ഗണ്യമായി മാറുന്നു എന്നാണ്. ഇതിന്റെ ഫലമായി, കൊതുകുകളുടെ ഗന്ധം തിരിച്ചറിയാനുള്ള കഴിവിൽ മാറ്റങ്ങൾ വരാം.,

പെൺ കൊതുകുകളുടെ പ്രായം കൂടുമ്പോൾ, മുട്ടകളുടെ വളർച്ചയ്ക്കായി രക്തം തേടുന്ന പെരുമാറ്റത്തിലേക്ക് അവ ക്രമേണ മാറുന്നു. ഈ സമയത്ത്, കൊതുകിന്റെ ആന്റിനകളിലെ ഗന്ധം തിരിച്ചറിയുന്നതിനാവശ്യമായ പല ജീനുകളുടെ പ്രകടനവും വർദ്ധിക്കുന്നതായി കാണപ്പെടുന്നു. എന്നാൽ, Or117 എന്ന പ്രത്യേക ഗന്ധഗ്രാഹി ജീനിന്റെ പ്രകടനം പ്രായം കൂടുന്നതിനനുസരിച്ച് കുറയുന്നു. പെരുമാറ്റപരവും നാഡീസംബന്ധവുമായ വിവിധ

പരീക്ഷണങ്ങൾ വഴി, Or117 ജീനും അതുമായി ബന്ധപ്പെട്ട പുഷ്പഗന്ധ ഘടകവും, പ്രായം കുറഞ്ഞ പെൺ കൊതുകുകളെ തേൻ ഉറവിടങ്ങളിലേക്ക് ആകർഷിക്കാൻ നിർണായകമാണെന്ന് കണ്ടെത്തിയിട്ടുണ്ട്.

ഗന്ധജ്ഞാനത്തിന്റെ ഈ വഴക്കം (പ്ലാസ്റ്റിസിറ്റി) കൊണ്ടാണ് കൊതുകുകൾക്ക് ആന്തരികവും ബാഹ്യവുമായ മാറ്റങ്ങൾക്ക് അനുസരിച്ച് പെരുമാറ്റം ക്രമീകരിച്ച്, അതിവേഗം പരിസ്ഥിതിയിലേക്ക് മാറാനുള്ള കഴിവ് കൈവരുന്നത്..

Acknowledgements

If you have seriously read or simply flipped through these pages, thank you either way. The journey contained within these pages is a result of not a solitary effort but a collective endeavour with the support of many.
To my supervisory team, thank you for setting me on this path 4 years ago!

Rickard, my deepest gratitude for being a source of inspiration both as a scientist and a supervisor. Your meticulous attention to detail has set a standard I aspire to, and your insightful feedback has been a guiding force throughout my journey. Thank you for your trust, kindness, clarity, and steady support.

Sharon, thank you for being a constant source of encouragement and guidance. Thank you for inspiring me and for being critical, yet kind with feedback. Your support has lifted me from several lows.

Silke, I am grateful for your valuable feedback and for the warmth and kindness extended to me, especially during my time in Jena. You always made me feel welcome.

Paper III is very special to me. What started off as a side project quietly grew into something much bigger. It spanned almost three years and carried me threw some of my lowest moments, alone in the dark room, watching mosquitoes! Seeing it finally come to a full story has been wonderful. To all co-authors: Thank you!
Julien, your patience and generosity while guiding a molecular biology novice (me!) didn't go unnoticed. Anaïs, though we never met in person, your work and thoughtful correspondence carried this forward, thank you!. Beti, your kindness and our many conversations were always a comfort. Gabi, thank you for always receiving me with such warm and genuine enthusiasm. Lukas, thank you for both great science and genuine friendship.

To everyone (former and current), in the Disease Vector Group and Unit of Chemical Ecology, thank you for building a space where work felt easier, questions were welcomed, and support was always within reach. I am so grateful for the time spent and friendships built. Special thanks to Elin and Mengistu for your everyday kindness and for helping me find things and at times, perspective when I needed them.

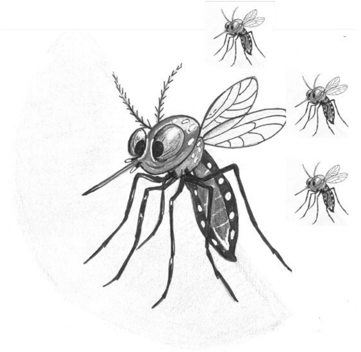
To Annika and Rohan, so grateful for your valuable feedback on the texts and for your constant support and friendship.

To everyone at nGICE, I am so glad to have been part of this group. Thank you for the shared science, thoughts, and friendships that emerged along the way.

To everyone at MPI, Jena, thank you for making me feel at home. Benjamin, thank you for your time, kindness and patience in teaching me brain dissections. Bill, thank you for your openness, support and guidance.

To the friends who stood by me, thank you for grounding me. For being the quiet in the chaos, the laughter on a long day, a reminder of life outside lab. Here's to more conversations, dancing, music and love.

And to my family, Acha, Amma and Abhi, thank you for your unconditional love. Thank you for always telling me "I can", especially on the days I couldn't see forward. Thank you!



To dear mosquitoes, who kept me company on the days that felt long and uncertain, thank you!

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Effect of extended egg quiescence and elevation in carbon dioxide on life history traits of *Aedes aegypti*

Sukritha Nalikkaramal[1,2], Sharon Rose Hill[1,2] & Rickard Ignell[1,2]

Elevation in carbon dioxide is a global threat, driving anthropogenic climate change. How disease-vectoring mosquitoes respond to these changes is currently largely unknown. The dengue vector, *Aedes aegypti*, has adapted to urban environments, which are more affected by climatic changes, especially CO_2. *Aedes aegypti* lay eggs around ephemeral water bodies that are prone to desiccation, with the pharate larvae possessing the ability to resist the desiccation, during which the permeability across the chorion is compromised. The study investigates the combined effects of elevated atmospheric CO_2 and extended egg quiescence duration on life-history traits of immature and adult stages, including development rate, survival and size. Furthermore, we analysed the metabolic reserves of newly emerged females and whether mosquitoes display compensatory feeding in response to restricted reserves. Extended egg quiescence duration, combined with elevated CO_2 level, differentially affected developmental duration and larval survival, with carry-over effects on adult metabolic reserves, size and survival. The interaction of elevated CO_2 conditions and egg quiescence period differentially impact life-history traits of *Ae. aegypti*. The findings of this study provide evidential support for assertion that changing climatic conditions significantly impact survival and population dynamics, as well as feeding propensity, which directly affect the vectorial capacity of *Ae. aegypti*.

Keywords *Aedes aegypti*, Carbon dioxide, Climate change, Egg quiescence, Feeding, Life-history, Metabolic reserves

Global climate change, due to anthropogenic activities, is predicted to change the distribution of insects, including mosquitoes that vector disease, across spatial and temporal scales[1–3]. A major driver of this predicted change is the elevation in atmospheric carbon dioxide (CO_2)[4]. Since the industrial revolution, the average CO_2 level has almost doubled (420 ppm)[5], and is predicted to increase to 600 ppm by 2050 and 1000 ppm by 2100[6]. The increase in global CO_2 levels to date has led to a significant reduction in pH through acidification of oceans[7,8] and freshwater bodies[9,10] which adversely affects the residing organisms. Despite the growing concerns regarding the expanding geographic distribution of disease-vectoring mosquitoes, such as the primary vector of dengue, yellow fever, chikungunya and Zika, *Aedes aegypti*[11–15], which can adapt to and occupy various ecological niches, little is known about how predicted elevation in CO_2 levels will affect life history traits[2,16]. Such information may increase our understanding of the factors affecting population dynamics[17] and feeding avidity, which intrinsically regulate vectorial capacity[18,19].

Breeding water bodies of mosquitoes present both biotic and abiotic stresses that influence the survival and development of the immature stages[20]. For example, *Aedes aegypti* lay eggs in ephemeral water bodies that are prone to desiccation. As a result, the eggs have evolved to withstand periods of dormancy and desiccation, through egg quiescence[21,22], the duration of which is regulated by environmental factors, such as temperature and humidity[20,23,24]. During the egg quiescence period, the larva depends on the maternal reserves for survival, and will hatch when favourable conditions arise[25]. Extended egg quiescence duration has been shown to affect the permeability of the chorion, as well as larval susceptibility to abiotic stressors, ultimately affecting adult fitness[26,27]. Weather conditions related to climate change, including warmer, wetter and drier conditions, have been demonstrated to affect larval hatching, survival and development, as well as adult fitness[28,29]. Moreover, water chemistry is known to affect life history traits, either directly[27,30–32] or indirectly[33–35]. For example, elevated CO_2 levels can reduce larval survival and increase developmental duration, as well as affect leaf litter decomposition[29], although contradictory data has also been reported[34,35]. While elevation in CO_2 has been shown to affect the physiology and behaviour of other freshwater-dwelling invertebrate life forms, including

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daphnia[8,36,37], mussels[38,39] and midget larvae[37], their effect on mosquito life-history traits remain poorly understood.

This study investigated how extended pharate larval quiescence duration and elevated CO_2 levels affect the life history traits of larval and adult stages of *Ae. aegypti*, including larval survival and developmental duration, as well as the carry-over effects on adult survival and body size. Additionally, the teneral metabolic reserves of females and their resource-feeding behaviour were assessed. The findings expand our understanding of how extended egg quiescence and elevation in CO_2 levels interact to affect the development and survival of both immature and adult stages. These stress factors also affect the teneral reserves of female mosquitoes, regulating their feeding behaviour, which could have important implications for vectorial capacity.

Results

Developmental duration of immature stages and larval survival

Elevation in CO_2 level and the extent of egg quiescence significantly varied the developmental duration of immature stages from larvae to pupae (Kruskal–Wallis test; $p < 0.0001$; Fig. 1a). The developmental duration of immature stages, originating from 2-week-old eggs, was significantly reduced when reared under 1000 ppm CO_2 condition (Fig. 1a). In contrast, the developmental duration of immature stages, originating from older eggs, increased significantly when reared under 600 ppm and 1000 ppm, compared to ambient CO_2 condition (Fig. 1a).

The probability of larval survival varied significantly with an interaction between the level of CO_2 (2-week-old eggs: Analysis of deviance, $\chi^2 = 21.54$, $p < 0.001$; older eggs: $\chi^2 = 28.0$, $p < 0.0001$, Fig. 1b) and extended egg quiescence (Analysis of deviance, $\chi^2 = 1540.4$, $p < 0.0001$, Fig. 1b). The survival probability of larvae originating from 2-week-old eggs reduced significantly at 1000 ppm CO_2, whereas larvae reared under 600 ppm had a similar survival probability as those reared under ambient CO_2 level (Fig. 1b). Similarly, the survival probability of larvae originating from older eggs, decreased when reared under 1000 ppm $CO_{2,}$ as well as 600 ppm $CO_{2,}$ compared to those reared under ambient CO_2 condition (Fig. 1b).

Effect on adult starvation tolerance and size

Adult starvation tolerance varied significantly with the increase in CO_2 level (2-week-old eggs: Analysis of deviance, $\chi^2 = 365.7$, $p < 0.0001$; older eggs: Analysis of deviance, $\chi^2 = 149.8$, $p < 0.0001$; Fig. 2) and extended egg quiescence, with an interaction between the two factors (Analysis of deviance, $\chi^2 = 150.5$, $p < 0.0001$; Fig. 2). The starvation tolerance of females originating from 2-week-old eggs, increased at 600 ppm CO_2, while starvation tolerance of both males and females reduced at 1000 ppm CO_2, compared to adults reared under ambient CO_2 conditions (Fig. 2), with significant differences observed between the sexes (Analysis of deviance, $\chi^2 = 171.1$; $p < 0.0001$; Fig. 2). In contrast, the starvation tolerance of females and males originating from older eggs was significantly different from each other (Analysis of deviance, $\chi^2 = 405$; $p < 0.0001$; Fig. 2), and increased when reared under 1000 ppm CO_2, when compared to 600 ppm CO_2 and ambient CO_2 conditions (Fig. 2).

Adult size varied significantly with the increase in CO_2 level and extended egg quiescence, (Kruskal–Wallis test; $p < 0.0001$; Supplementary Figure S1). The effect of elevated CO_2 levels and extended egg quiescence was female-specific (Supplementary Figure S1), where the body size of females originating from 2-week-old eggs significantly decreased in response to an elevation in CO_2 level (Supplementary Figure S1). Contrastingly, the size of females originating from older eggs did not differ across CO_2 conditions and were significantly smaller than females originating from 2-week-old eggs, when reared under 600 ppm and ambient CO_2 conditions (Supplementary Figure S1).

Effect on total energy reserves

Teneral metabolic reserves of individual females were analysed to quantify the total content of carbohydrates, glycogen, lipids and proteins accumulated during the aquatic stage in response to extended egg quiescence duration and elevated CO_2 levels (Fig. 3). The soluble carbohydrate content varied significantly in response to both egg quiescence duration and CO_2 conditions (Kruskal–Wallis test; $p < 0.0001$; Fig. 3a). The soluble carbohydrate content of females, irrespective of egg quiescence duration, was significantly lower when reared under 1000 ppm CO_2 condition (Fig. 3a). The content of glycogen, which is a stored form of carbohydrate, differed significantly between females in response to egg quiescence duration and CO_2 conditions (Kruskal–Wallis test; $p = 0.0003$; Fig. 3b). The glycogen content of females originating from 2-week-old eggs was significantly lower in response to elevated CO_2 conditions, as opposed to the glycogen content of females originating from older eggs, that was not affected by CO_2 conditions (Fig. 4b). The lipid content in females differed significantly in response to egg quiescence and CO_2 conditions (Kruskal–Wallis test; $p < 0.0001$; Fig. 3c). The only significant pairwise comparisons were observed between females originating from older eggs, reared under 1000 ppm CO_2 level, in which the lipid content was lower compared to the counterparts reared under 600 ppm and ambient CO_2 conditions (Fig. 3c). The total protein content of females differed significantly in response to egg quiescence period and CO_2 conditions (Kruskal–Wallis test; $p < 0.0001$; Fig. 3d). While the total protein content of females, originating from either egg quiescence conditions, remained similar across the CO_2 conditions, females originating from older eggs had higher protein content compared to females originating from 2-week-old eggs when reared at 600 ppm CO_2 (Fig. 3d).

Feeding response of teneral females

No-choice feeding assays (Fig. 4A) were conducted to assess a potential compensatory feeding response by newly emerged female mosquitoes, as a consequence of the carry-over effects resulting from the stress caused by extended egg quiescence duration and elevated CO_2 conditions. The proportion of females, which originated from 2-week-old eggs, that fed on either honey (Fig. 4a, left) or blood (Fig. 4a, right), was not significantly different

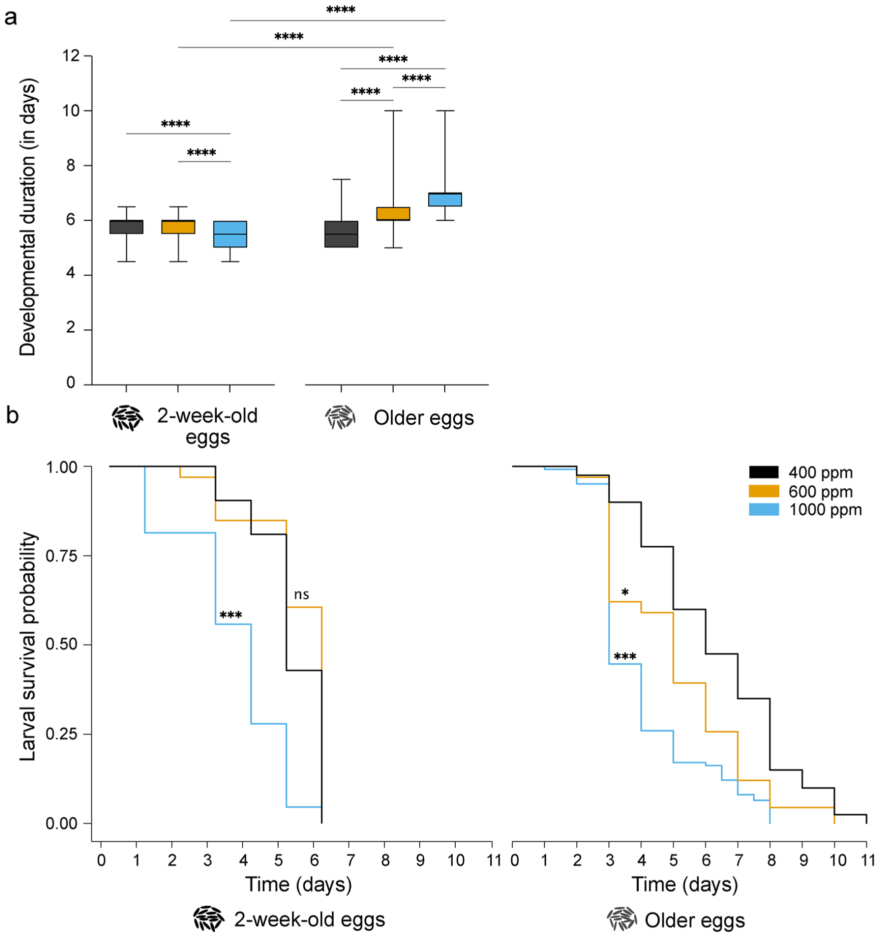

Fig. 1. The effect of extended egg quiescence and elevated CO_2 levels on immature stage development and survival. (**A**) Developmental duration of the immature stage from larvae to pupae. For comparison between groups, a Kruskal–Wallis test followed by Dunn's multiple comparisons test was performed. The whiskers denote minimum to maximum, and asterisks indicate significant differences between the groups ($N = 3$, $n = 300$ larvae, $p < 0.05$). (**B**) Survival probability of the larvae originating from 2-week- and 3–6 months-old (older) eggs. The curves were analysed using a Cox regression model, followed by a log-rank *post-hoc* test using the 'survival' package ($N = 3$, $n = 300$ larvae, $p < 0.05$).

between CO_2 conditions. In contrast, females originating from older eggs fed significantly and proportionately less on honey with an increase in CO_2 level (Fig. 4a, left). Moreover, the proportion of females emerging from older eggs, feeding on blood, was significantly reduced when reared under 1000 ppm CO_2 compared to those reared under 600 ppm CO_2 condition (Fig. 4a, right), as well as in comparisons with females emerging from 2-week-old eggs reared under 1000 ppm CO_2 condition (Fig. 4a, right).

Colorimetric analysis was performed to quantify the volume that the females imbibed during the differential feeding on honey (Fig. 4b, left) and blood (Fig. 4b, right). Females originating from 2-week-old eggs reared at 1000 ppm CO_2 imbibed a significantly lower volume of honey compared to females reared under 600 ppm and ambient CO_2 conditions (Fig. 4b, left). In contrast, females originating from older eggs, irrespective of CO_2 conditions, imbibed a similar volume of honey, while those reared from the younger eggs imbibed less at 1000 ppm CO_2 (Fig. 4b, left). Females originating from both 2-week-old and older eggs, irrespective of CO_2 conditions, generally imbibed a similar volume of blood (Fig. 4b, right). However, females originating from older

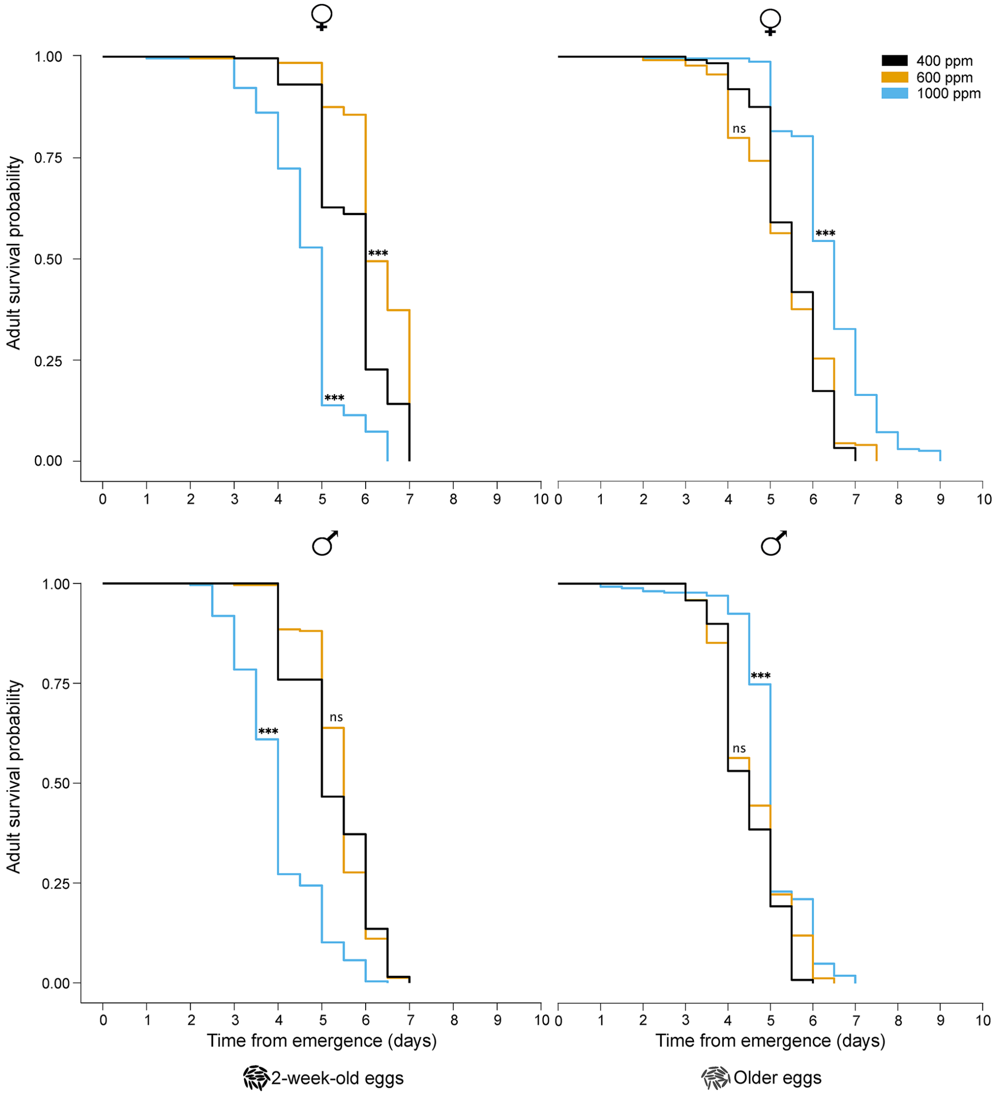

Fig. 2. The interactive effect of extended quiescence and elevation in CO_2 levels on the survival of adult *Aedes aegypti*. Survival probability curves of the adults are separated by sex and egg quiescence period. The curves were analysed using a mixed-effects Cox regression model, followed by a log-rank *post-hoc* test using the 'survival' package (n (per group) = 245–250, $p < 0.05$).

eggs reared under ambient CO_2 conditions, generally imbibed significantly higher volumes of blood compared to females reared under other CO_2 conditions (Fig. 4b, right).

Effect of artificial manipulation of water acidity

The effect of artificially manipulated larval water pH was assessed on immature development duration, survival and adult starvation tolerance (Fig. 5). Artificial manipulation of water acidity did not have a significant effect on immature development duration (Kruskal–Wallis test; $p = 0.90$ Fig. 5a), larval survival (Analysis of deviance,

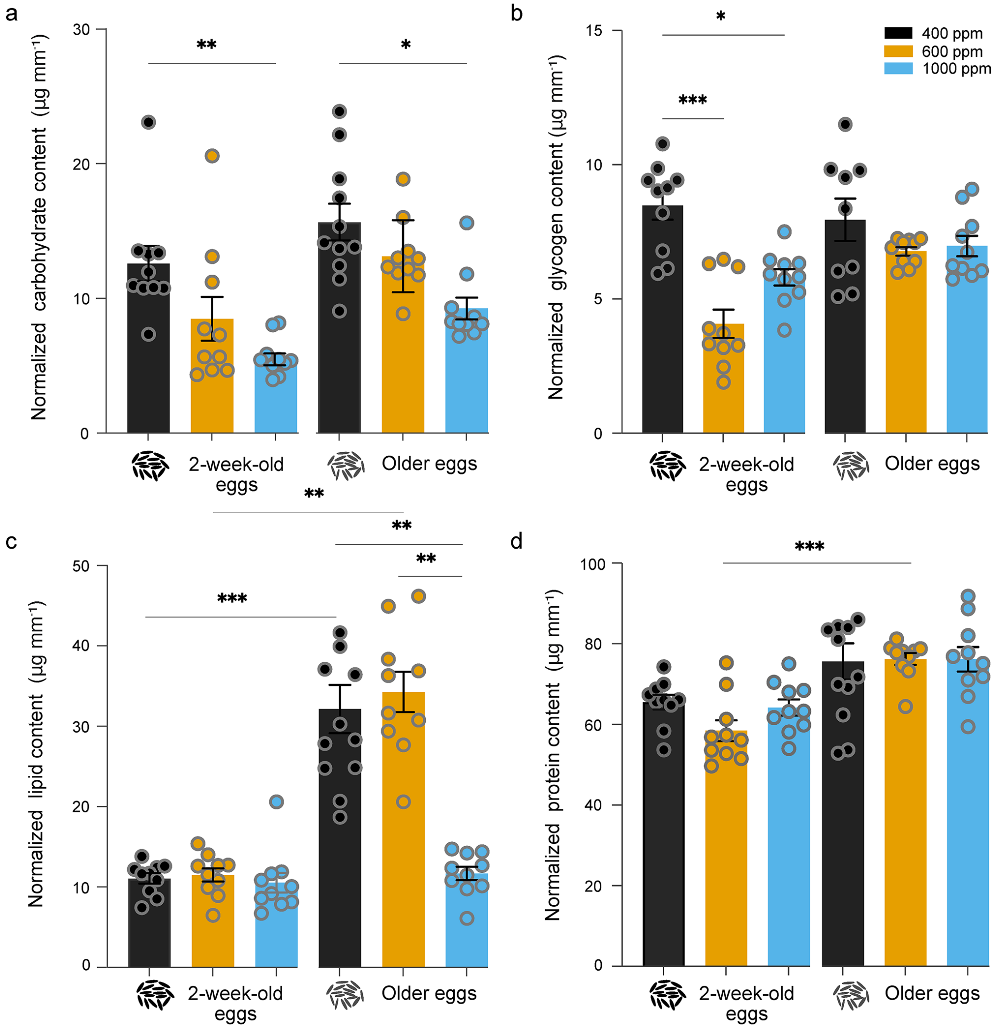

Fig. 3. Metabolic reserves accumulated by teneral females in response to elevated CO_2 levels and egg quiescence duration. The amount of soluble (**A**) carbohydrate, (**B**) glycogen, (**C**) lipid and (**D**) protein content normalized for body size. The error bars represent the standard error of the mean, and asterisks denote the significant differences between the groups. For comparison between groups, a Kruskal–Wallis test followed by Dunn's multiple comparisons test was performed ($n = 10$, $p < 0.05$).

$\chi^2 = 1.05$; $p = 0.31$ Fig. 5b) or adult starvation tolerance (Females: Analysis of deviance; $\chi^2 = 1.78$; $p > 0.05$, Males: Analysis of deviance; $\chi^2 = 4.38$; $p > 0.05$; Fig. 5c) when compared to ambient CO_2 condition.

Discussion

The atmospheric CO_2 level is predicted to increase up to 1000 ppm within the next century. This increase, and even that predicted within a shorter period of time, significantly affects several life-history traits and feeding response of *Ae. aegypti*, an affect modulated by the extent of egg quiescence duration. While elevated CO_2 conditions and extended egg quiescence duration negatively affected the aquatic stages, the carry-over effects to adults were differential. We present our findings below, in the context of what is known about the effects of CO_2

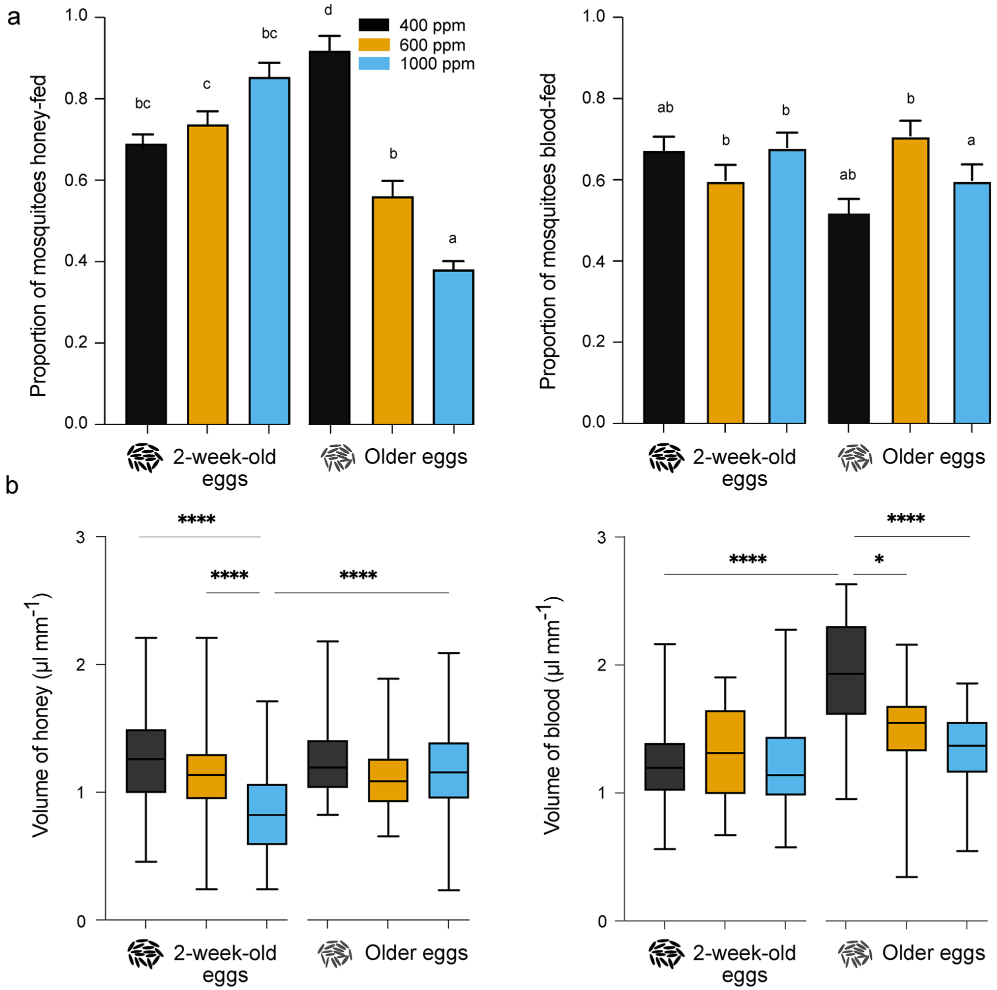

Fig. 4. Differential feeding of female *Aedes aegypti* in response to elevated CO_2 conditions and extended egg quiescence duration. (**A**) The proportion of teneral females that fed on honey (left) and blood (right) was differentially and significantly affected by extended egg quiescence duration and elevation in CO_2 level. The bars represent mean (± SE) of proportion of females feeding and letters denote significant differences in pairwise comparisons using 'emmeans' Tukey method ($n = 160–170$ females, $p < 0.05$). (**B**) Volumetric analysis of imbibed honey (left) and blood (right) normalised for body size. For comparison between groups, a Kruskal–Wallis test followed by Dunn's multiple comparisons test was performed. The whiskers denote the minimum to maximum values and asterisks indicate the significant differences between the groups ($n = 50$ females, $p < 0.05$).

and other climatic factors on mosquito life history traits, and what consequence this may have for population dynamics and vectorial capacity.

Elevation in CO_2 directly and differentially affects larval development duration[33] and survival, as well as adult survival and size, an effect that is dependent on the extent of egg quiescence duration. Whereas the negative effects of elevated CO_2 levels observed in this study is in line with previous studies on other aquatic organisms[9,37,39,40], the positive effect on adult survival has not been previously reported. When reared under elevated CO_2 conditions, larvae that emerge from an extended egg quiescence duration had a delayed developmental duration. As pharate larvae depend on maternally-derived reserves, an extended egg quiescent duration leads to reduced

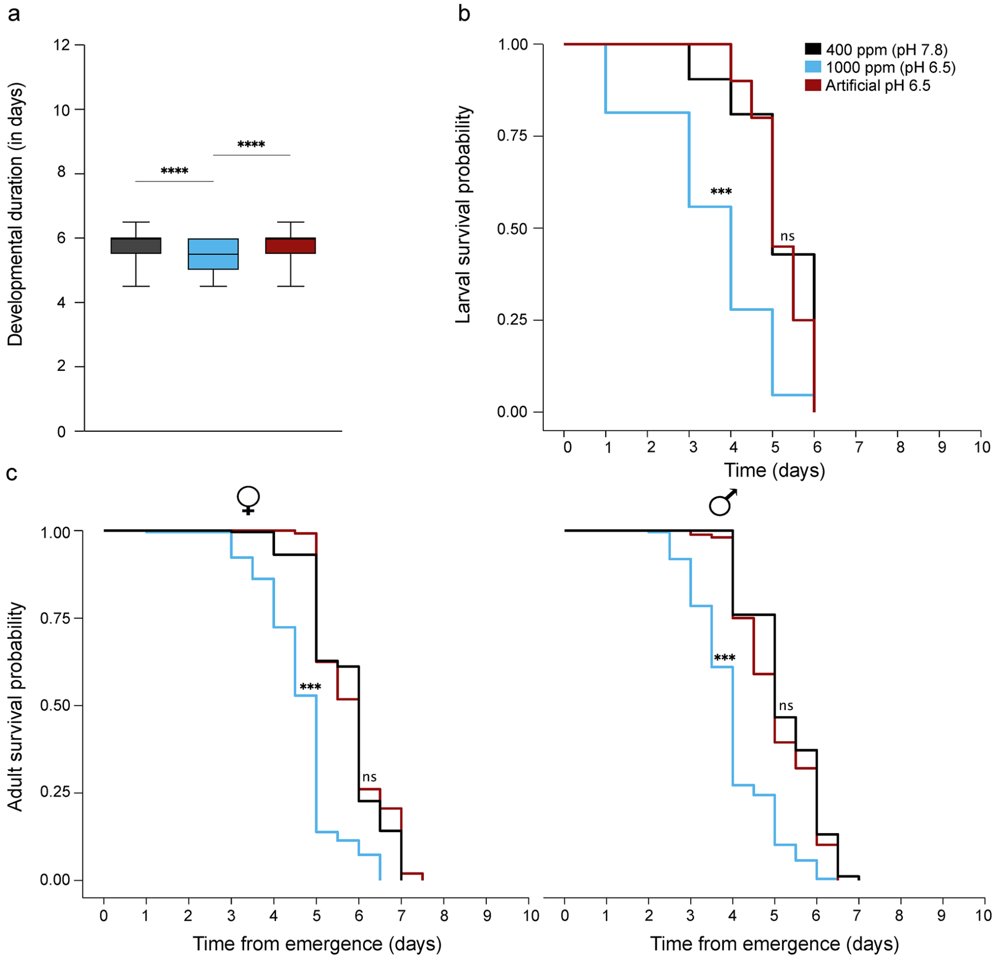

Fig. 5. The effect of water acidification on the life history parameters of immature and adult stages of *Aedes aegypti*. (**A**) Developmental duration of the immature stage from larvae to pupae. For comparison between groups, a Kruskal–Wallis test followed by Dunn's multiple comparisons test was performed. The whiskers denote minimum to maximum values, and asterisks indicate significant differences between the groups (N = 3, n = 300 larvae, $p < 0.05$). (**B**) Survival probability of the larvae originating from 2-week-old eggs reared under ambient and 1000 ppm CO_2 conditions, as well as at pH 6.5. (**C**) Adult survival probability curves are separated by sex. The curves were analysed using a mixed-effect Cox regression model, followed by a log-rank *post-hoc* test using the 'survival' package (n = 245–250, $p < 0.05$). The data shown for ambient (pH 7.8) and 1000 ppm (pH 6.5) CO_2 are the same as in Figs. 1 and 2.

energy reserves, increasing the vulnerability of larvae to sub-optimal or stressful conditions[27]. This limitation in energy reserves likely contribute to the significant reduction in survival of larvae emerging from an extended egg quiescence duration in response to elevated CO_2 conditions, compared to those emerging from newer eggs, by negatively impacting the homeostasis[41]. Similar observations of higher mortality and delayed growth following exposure to elevated CO_2 in copepods were attributed to additional energy demands[42]. To compensate for limited energy reserves upon emergence, larvae need to accumulate carbohydrate and lipid reserves, by feeding on, *e.g.*, detritus, to allow them to metamorphose into pupae[43]. This could explain the observed delay in developmental duration of larvae that emerged from an extended egg quiescence, at elevated CO_2 conditions. Similar observations have been observed in mosquitoes and other aquatic organisms in response to various abiotic and biotic environmental stressors, including temperature, photoperiod and larval density[44–46]. An increased developmental time, not only increases foraging, thereby increasing the risk of predation, but also exposes the aquatic stages to habitat changes, such as drought[20]. In contrast to larvae emerging from extended egg quiescence duration, the larvae emerging from new eggs and reared at 1000 ppm CO_2 had a significantly shorter development duration, which could be a defence strategy, in which larvae with higher maternal reserves pupate earlier and escape the stressful larval habitat[47]. Thus, larvae emerging from shorter or extended quiescence duration appear to have different adaptation strategies to environmental stress. These findings highlight the importance of considering the interactive effects of climatic factors, which play a critical role in influencing immature stage development and survival[47,48], this study.

An elevation of CO_2 in stagnant freshwater bodies cause physiological stress in aquatic organisms[39,49,50], including mosquito larvae[35], this study, through acidification of either the bodily fluids or the water[30,51], or hypercapnia[49,52]. While elevation in CO_2 can cause weak acidification in freshwater ecosystems[10], water acidification in the absence of CO_2 had no effect on the life history parameters of *Ae. aegypti* [this study], similar to what has been observed in *Daphnia*[9] and freshwater zooplankton[36]. Hypercapnia-induced narcotic effects and associated effects on survival have been studied in other invertebrates[49,53] and fish[54]. The mode of action of CO_2, elevated to levels that reflect predicted changes, as used in this study, and how these affect the observed life history parameters, is unclear and requires further study.

Larval environmental parameters dictate the carry-over effects to emerging adults[55–58], with significant effects on survival, size and reproductive success, as well as vectoral capacity[59]. Elevated CO_2 levels during the aquatic phase significantly and differentially affected the survival and size of emerging males and females, an affect modulated by the interaction between egg quiescence duration and larval development duration. Similar interactive effects of environmental parameters with other abiotic stressors have been reported across other mosquito species[47,60]. The seemingly counterintuitive higher starvation tolerance in adults, following rearing at elevated CO_2 conditions, could be indicative of metabolic priming, *i.e.*, adults emerging from stressful larval environments display anticipatory priming on their metabolic reserves[61,62], which requires further investigation. We hypothesise that this metabolic priming likely obscures the correlation between metabolic reserves in teneral adults and survival, with body size having no significant effect.

Teneral mosquitoes differentially metabolise lipids or glycogen into carbohydrates depending on experienced egg quiescence period and CO_2 conditions, but the low levels of accumulated reserves are not offset by compensatory feeding on either honey or blood. The glycogen and lipid content in teneral females originating from 2-week-old or older eggs, respectively, was significantly lower at elevated CO_2 levels. Teneral females that originated from different egg quiescence periods appear to employ different metabolic strategies to cope with environmental stress, likely regulated at the metabolic enzyme activity level[62], which requires further investigation. Similar to lipids and glycogen, the carbohydrate content in females was significantly low in response to the two stress factors, suggesting low energy reserves for locomotion during non-feeding periods[63]. While an expected response to this would be an increased compensatory feeding on either honey or blood, which is often used as an energy resource by nutritionally deprived mosquitoes[20] the opposite was demonstrated, likely due to metabolic priming during the larval stage. Alternatively, nutritionally deprived mosquitoes may not be sufficiently motivated to spend energy to seek energy under the current conditions.

The vectorial capacity of mosquitoes hinges directly on the life history traits of the aquatic and adult stages, as well as the propensity of adult females to feed on human hosts. A slight reduction in development duration and survival in response to climatic change, although subtle, may have a significant and differential effect on mosquito population dynamics, which needs to be considered in future models. Moreover, these models need to take into consideration the effect of metabolic priming on starvation tolerance, and how elevated CO_2 in combination with other climatic factors affect feeding patterns. Future semi-field and field studies will be required to further elucidate the effects of elevated CO_2 on the life history trait of disease vectoring mosquitoes.

Materials and methods

Rearing of Aedes aegypti

For colony maintenance, *Ae. aegypti* (Rockefeller) were reared at 27 ± 2 °C and 65 ± 5% relative humidity, and a 12 h: 12 h light: dark cycle. Adult mosquitoes were provided ad libitum access to 10% sucrose solution, and females were allowed access to sheep blood (Håtunalab AB, Bro, Sweden), in a 1.5 ml reservoir covered with a collagen membrane using a membrane feeding system (Hemotek Ltd, Blackburn, UK), for egg production. Blood-fed females were given access to a wet conical filter paper placed above plastic cups filled with distilled water. Eggs laid on filter paper until 48 h were collected, labelled and stored in the rearing chamber until further use.

Carbon dioxide acclimatisation

For the experiments, three high-precision climate chambers (ca 11.5 m^2 with a free height of 2.3 m) were used, in which temperature, humidity and light were maintained as in the main rearing. The CO_2 concentration in the chambers was set to ambient (ca. 400 ppm), 600 ppm and 1000 ppm, respectively, delivered through cylinders containing pure CO_2 (Strandmöllen, Ljungby, Sweden), and regulated by the climate system. A filter paper containing age-controlled eggs (2-week and 3-to-6-month quiescent periods, respectively) from the main rearing was divided into approximately three equal parts and transferred to each of the climate chambers. The eggs were then placed in plastic larval trays (24 cm × 17.5 cm × 8 cm) filled with water (600 ml), previously acclimatised in the chambers for 48 h. A pinch of fish food (TetraMin® Flakes, Melle, Germany) was added to each tray to stimulate hatching[24]. The larvae that hatched within 18 h were divided into individual larval trays, with a density of 100 larvae in 600 ml of water. The larvae were fed fish food daily (1 mg $larvae^{-1}$) to provide favourable conditions to maximise life-history parameters and reduce competition[47,64,65], with food quantity adjusted to account for a reduction of larvae due to mortality. The water was changed every second day to control for microbial growth and accumulation of debris.

Immature development and survival

Developmental duration, *i.e.,* the time from egg hatching to pupation, was assessed from observations done every 12 h, and differences between treatments analysed using a non-parametric test, Kruskal–Wallis test followed by Dunn's multiple comparison test for select comparisons (GraphPad Prism, for Macbook 10.0.0 (131)). The pupae were collected every 12 h and transferred into small plastic cups with distilled water, and placed in Bugdorm cages (17.5 cm × 17.5 cm × 17.5 cm; Megaview Science Co., Ltd, Taichung, Taiwan) for further analysis of adult life history parameters. The larval survival probability was estimated by counting the number of live larvae every 12 h until all the larvae either pupated or died. Three independent replicates, each with 100 larvae, were conducted for each treatment and repeated thrice (Supplementary Figure S2). A mixed-effect Cox regression survival model was used to analyze the effect of elevated CO_2 levels and egg quiescence on larval survival, with the replicate number and larval tray as fixed variables. A *post-hoc* test was then performed separately for the two egg quiescence periods with a log-rank test using the 'survival'[53] package in RStudio[54].

Adult starvation tolerance and size

Survival assays were conducted to assess adult starvation tolerance as a consequence of the metabolic reserves carried over from the immature stages. Emerging adult mosquitoes were provided access to distilled water, and the number of dead mosquitoes monitored every 12 h until all the mosquitoes in a cage were dead. To limit competition, each cage contained not more than 50 adult mosquitoes. A mixed-effect Cox regression survival model was used to analyze the effect of elevated CO_2 levels and egg quiescence on adult survival with replicate number and cage as the fixed variable. A *post-hoc* test was then performed separately for the egg quiescence period and sexes with a log-rank test using the 'survival'[66] package in RStudio. For adult body size, the right wing of individual male and female mosquitoes was dissected under a stereomicroscope, and the distance from the axillary incision to the apical margin, excluding the wing fringes[68], was measured using an ocular micrometre. For the analysis, Kruskal–Wallis test was performed followed by Dunn's multiple comparison test for select comparisons (GraphPad Prism, for Macbook 10.0.0 (131).

Estimation of teneral metabolic reserves

The teneral metabolic reserves were analysed by quantifying the carbohydrate, glycogen, lipid and protein content of individual adult female mosquitoes. For the analysis, adult females (up to 12 h post-emergence) were freeze-killed and stored in 2 ml Eppendorf tubes at -20 °C. The biochemical analysis was done according to van Handel's calorimetric estimation methods modified by Foray et al.[69]. Protein analysis was performed according to Bradford's method[70], using the Bio-Rad Protein Assay Kit II (Bio-Rad Laboratories, Inc., Copenhagen, Denmark) with bovine serum albumin as a standard. Total carbohydrate and glycogen analyses were performed using anthrone (CAS: 90448, Sigma-Aldrich, Stockholm, Sweden) prepared in 95% sulphuric acid, with D-glucose (1 mg ml^{-1}) as a standard[71]. Total lipid analysis was performed following a chloroform–methanol step, using vanillin (CAS: 121335, Sigma-Aldrich), prepared in 85% phosphoric acid, with olive oil (1 ml ml^{-1}) as a standard[72]. The absorbance for the total carbohydrates (carbohydrate and glycogen), lipids and protein analyses were measured in 96 well plates at 625 nm, 525 nm and 595 nm, respectively, using a microplate reader (Multiskan ™ FC Microplate Photometer**,** Thermo Scientific™, Stockholm, Sweden). The content of carbohydrate, glycogen, lipid and protein was calculated based on comparisons with standard curves, adjusted for the dilution factor, and normalised for the mean wing size of the mosquito. For the experiment, ten females were randomly analysed for each treatment group. A comparison of medians was conducted with Kruskal–Wallis test followed by Dunn's multiple comparisons test for select comparisons (GraphPad Prism, for Macbook 10.0.0 (131)).

Feeding assays

No-choice feeding assays were conducted to correlate hypothesised compensatory feeding of females due to constraints posed by the metabolic reserves. Mosquitoes (24 h-to-48 h post-emergence) were aspirated into BugDorm cages in groups of 20-to-25 individuals per cage, and starved for 24 h with ad libitum access to water until 2 h prior to the start of the experiments. Experiments were conducted during the peak activity of the mosquitoes at Zeitgeber time 9–12[73], in the respective Biotron chambers. To assess the proportion of mosquitoes feeding and volume imbibed, all experiments were repeated six times, including two replicates of each egg batch. In addition, 20–25 mosquitoes from the same egg batch were provided access to water, and were used as controls for the volumetric analysis. After feeding, mosquitoes were carefully placed into 1.5 ml Eppendorf tubes and immediately frozen at-20 °C until further analysis. For the feeding proportion comparison, a generalised linear model with a binomial distribution followed by a *post-hoc* pairwise comparison with Bonferroni p value correction performed using the 'emmeans' package in RStudio[67].

Honey (60% honey, prepared in distilled water) was used as the carbohydrate-rich source. To quantify the volume of honey imbibed, 1 mg ml^{-1} xylene cyanole (FF; CAS 2650–17-1; Sigma-Aldrich) was added, and fresh solutions prepared on the day of experiments. Mosquitoes were given access to the honey solution for 3 h. To quantify the volume imbibed, 230 µl of distilled water was added to the Eppendorf tubes containing the females provided with the honey solution. The tissues were then homogenised using a disposable pestle attached to a cordless motor (VWR®, Lund, Sweden), and then centrifuged at 6720 rcf for 10 min. The supernatant (200 µl) was transferred into individual wells of 96-well microplates (Sigma-Aldrich), and the absorbance measured at 620 nm using a spectrophotometer-based microplate reader. A standard curve was generated by preparing serial dilutions from 0.1 µl to 2.4 µl of 1 mg ml^{-1} xylene cyanol, and used to determine the volume of honey imbibed by each mosquito.

Sheep blood was used as the protein source, which was provided to the females for 1 h using the artificial membrane system described above. To quantify the volume of blood imbibed, the haemoglobinometry method[74] was used. The abdomens of fed and unfed were dissected and homogenised in Drabkin's reagent (prepared as detailed in[74]), followed by the addition of chloroform. The supernatant (200 µl) was pipetted into individual wells of 96-well microplates, and the absorbance measured at 540 nm using a spectrophotometer-based microplate reader. The absorbance of unfed mosquitoes was used as a control and subtracted from the absorbance of the blood-fed individuals. To determine the individual volume imbibed, a standard curve was generated using different volumes of blood. For the volumetric analysis, a comparison of medians was conducted with Kruskal–Wallis test followed by Dunn's multiple comparisons test for select comparisons (GraphPad Prism).

Artificial manipulation of water acidity

To determine the effect of change in acidity on life history parameters, the pH of the larval water used in this experiment was decreased to the level measured in the larval water maintained under 1000 ppm CO_2, by the addition of 0.1 ml 0.1 N hydrochloric acid[8]; the pH was monitored throughout the experiment. Then, 2-week-old eggs were placed in the water and the life history traits of the emerging larvae and adults were assessed as described above.

Data availability

All data generated or analysed during this study are included in this published article.

Received: 11 July 2024; Accepted: 25 February 2025

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Acknowledgements

We acknowledge the help provided by the Biotron maintenance staff at SLU.

Author contributions

SRH and RI conceived the idea, SN, SRH and RI designed the methodology and SN collected and analysed the data. SN drafted the manuscript and all authors critically revised the manuscript. All authors gave final approval for publication.

Funding

Open access funding provided by Swedish University of Agricultural Sciences.

Sveriges Lantbruksuniversitet, Max Planck Centre,next Generation Insect Chemical Ecology.

Declaration

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-92193-4.

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Impact of elevated CO_2 level and egg quiescence duration on gene expression in the peripheral olfactory system of *Aedes aegypti*

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Elevation in CO_2 can significantly impact the biology of various organisms, affecting life-history traits of both aquatic and terrestrial forms, including disease-vectoring mosquitoes. For mosquitoes, this effect is accentuated by egg quiescence duration, resulting in a change in foraging of adult females. Female mosquitoes rely on their olfactory system for locating resources, such as nectar and blood. This study employs a transcriptomic approach to investigate how a projected elevation in CO_2 level, under a worst-case scenario, interacts with extended egg quiescence duration to modulate the molecular machinery of the peripheral olfactory system, the antennae and maxillary palps, of the yellow fever mosquito, *Aedes aegypti*. The transcriptome analysis demonstrates significant changes in the abundance of genes related to metabolism, xenobiotics degradation and chemosensory function, with the most pronounced effects observed in the CO_2 sensing tissue, the maxillary palp. The study provides novel insights into how anthropogenic climate change can modulate the olfactory sensory system of disease vectors, which may have cascading effects on resource-seeking behaviour.

Keywords Mosquitoes, Carbon dioxide, Climate change, Egg quiescence, Olfactory system, Transcriptome

Global climate change, due to anthropogenic activities, is predicted to change the distribution and behaviour of insects, including mosquitoes that vector disease[1–3]. A key factor driving this change is the elevation in atmospheric carbon dioxide (CO_2), which inadvertently affects life history traits across both aquatic and terrestrial stages of invertebrates[4–6]. For example, in the dengue vector, *Aedes aegypti,* an exponential increase in atmospheric CO_2 level, reflecting those projected within recent time and those predicted under extreme conditions, if targets are not met, for the next century[7], significantly affects key life-history traits, including larval survival and development, as well as adult survival and the feeding response of females[6]. These effects are further modulated by the extent of egg quiescence, *i.e.,* the ability of eggs to withstand extended periods of desiccation or dormancy[8], which is determined by environmental factors, such as temperature and humidity[9–11]. Prolonged egg quiescent duration increases the susceptibility of emerging larvae to abiotic stressors[12,13], which may have significant effects on mosquito population dynamics and feeding behaviour, thus affecting vectorial capacity[14]. The feeding response of insects is the ultimate stage in a process regulating resource seeking, which for most insects is mediated predominantly by olfaction and influenced by the internal physiological state[15,16]. Resource-seeking behaviours, as well as the detection of ecologically relevant sensory cues, in insects are affected by both short- and long-term exposure to elevated levels of CO_2[17–21].

Many insect species use CO_2 as a reliable cue for nectar[22,23], host[24,25], and oviposition site seeking[26], as well as threat avoidance[27,28]. An elevation in ambient CO_2 negatively affects host-seeking in mosquitoes[21] and oviposition site selection in moths, due to sensory constraints imposed on the CO_2-sensory system[21,26]. Long-term developmental exposure to elevated CO_2 also reduces the alarm-pheromone escape behaviour in aphids[29], although no significant effects on olfactory perception have been described[30]. In addition, studies on aquatic invertebrates show an impairment in olfactory-guided behaviours as a consequence of elevated CO_2[31], however, the underlying neuronal mechanism remains unclear[32]. Exposure to elevated CO_2 in *Helicoverpa* moths affects the CO_2-sensory neurons, which become promiscuous and respond to fluctuations in temperature, as well as to CO_2[33], demonstrating that exposure to elevated CO_2 likely has a broad effect on sensory systems and gene expression[34]. The aim of this study was to assess how predicted levels of elevated CO_2 and extended egg

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quiescence affect chemosensory gene expression to identify molecular correlates underlying changes in resource-seeking behaviours in female *Ae. aegypti*.

The antennae and maxillary palps constitute the main peripheral olfactory system of mosquitoes, with hair-like structures, sensilla, on the surface acting as the smallest functional units[35]. Volatile odorants enter the sensilla, where they are recognized and transported by odorant binding proteins (OBPs) and chemosensory proteins (CSPs) to receptors in the dendritic membrane of olfactory sensory neurons (OSNs)[36,37]. Each OSN expresses one or a combination of olfactory receptor proteins from three different families: odorant receptors (ORs), ionotropic receptors (IRs) and gustatory receptors (GRs)[38,39], as well as sensory neuron membrane proteins (SNMPs)[36]. The ORs and IRs form heterotetrameric complexes with conserved co-receptors, Orco, as well as Ir25a, Ir8a and Ir76b, respectively, and ligand-selective subunits, ORs and IRs[40,41]. The overall role of ORs and IRs in mosquitoes is to regulate host attraction and discrimination[42–45]. Although GRs are primarily involved in contact chemoreception, CO_2 is detected by a heteromeric complex of Grs[46,47], and involved in activation and attraction[48,49]. Apart from the canonical chemosensory gene families, pickpocket (PPK) and transient receptor potential (TRP) channels, involved in risk assessment[50–52], are also expressed in the OSN dendritic membrane[37]. Several members of these chemosensory gene families are differentially regulated in response to a change in internal state of female mosquitoes[16,53–55], however, there is currently limited information on how the external environment modulates the molecular machinery of the peripheral olfactory system.

To achieve the aim of this study, RNA sequencing was performed using antennal and maxillary palp tissues collected from females reared under current ambient and extreme CO_2 conditions and originating from eggs following different egg quiescent periods. The transcriptome analysis demonstrated an overall effect on the differential expression within select gene ontologies, including metabolism, xenobiotics and chemosensory, predominantly in the maxillary palp, in response to elevated CO_2 conditions, an affect exacerbated by egg quiescence duration. The findings of this study demonstrate that predicted changes in climate, driven by factors, such as elevation in CO_2, affect the peripheral olfactory system of insects, which in turn may affect the resource-seeking behaviours.

Results

RNA sequencing

The RNA sequencing detected a total of 17,439 genes of the 19,804 annotated genes in the genome of *Ae. aegypti*, of which 10,226 were reliably expressed (Supplementary Table S1). Of these, 8,833 and 9,510 were reliably expressed in the antennae and maxillary palps, respectively. To assess the quality and depth of the sequencing, the core eukaryotic gene mapping approach was performed, demonstrating that 450 and 447 (of the total 450) genes were detected reliably above the 1 TPM expression level in the antennal and maxillary palp libraries, respectively (Supplementary Table S2).

Overall and differential expression

Overall gene expression was assessed using Principal Component Analysis (PCA) with the 29 libraries of tissue collected from females reared under ambient and elevated CO_2 conditions, and shorter and extended egg quiescent periods (Fig. 1). The analysis revealed that 43.2% of the variance among libraries was based on the type of olfactory organ (PC 1), and 1.9% of the variance between maxillary palp libraries was based on CO_2 condition (PC 9) (Fig. 1). There was no significant effect on overall antennal gene expression in response to CO_2 level ($F = 1.01$, $R^2 = 0.064$, $p = 0.38$) or egg quiescent duration ($F = 1.68$, $R^2 = 0.10$, $p = 0.17$), individually or interactively ($F = 2.07$, $R^2 = 0.13$, $p = 0.09$, Supplementary Figure S1). In contrast, the egg quiescence period ($F = 3.43$, $R^2 = 0.20$, $p = 0.02$) significantly affected the overall gene expression in the maxillary palp (Supplementary Figure S1). However, neither CO_2 level ($F = 1.01$, $R^2 = 0.06$, $p = 0.38$) nor the interaction of the two stress factors ($F = 2.07$, $R^2 = 0.13$, $p = 0.09$) had a significant effect on the overall maxillary palp gene expression (Supplementary Figure S1).

The gene ontology (GO) analysis identified various molecular functional categories, based on differentially expressed genes (DEGs), which changed in both numbers and direction in the antennal and maxillary palp libraries in response to the interaction of an elevation in CO_2 and extended egg quiescence (Fig. 2). Comparisons between ambient and elevated CO_2, as well as between egg quiescence periods for antennal and maxillary palp libraries under ambient conditions, identified too few DEGs for drawing any overall findings (Fig. 2). In response to elevated CO_2 and extended egg quiescence, > 85% of the DEGs in the antennal and maxillary palp libraries were categorised as molecular function (GO:0,003,674), followed by oxidoreductase activity (GO:0,016,491), peptidase activity (GO:0,008,233) and hydrolase activity, acting on carbon–nitrogen (but not peptide) bonds (GO:0,016,810) (Fig. 2, right). In addition, in the maxillary palp libraries, the 1% DEGs were categorised as hydrolase activity, acting on glycosyl bonds (GO:0,016,798) (Fig. 2). Within the molecular function category, several differentially expressed chemosensory genes, including *Or*s, *Ir*s and *Obp*s, were represented.

The KEGG pathway analysis identified 39 unique metabolic pathway terms, 17 of which were from the metabolism pathways, 9 from biosynthesis of secondary metabolites, four from metabolism of terpenoids and polyketides, and nine identified in the xenobiotic biodegradation pathway (Fig. 3). In the antennal libraries, four DEGs were categorised as xenobiotic response pathway in females reared under ambient CO_2 conditions, in response to extended egg quiescence. Moreover, in the maxillary palp, six and 28 DEGs contributed to the xenobiotic biodegradation pathway, when reared under ambient and elevated CO_2, in response to extended egg quiescence (Fig. 3, Supplementary Table S3). Within the xenobiotic response pathway, several stress response genes, including cytochrome P450 and UDP-glycosyl transferases, were represented across the comparisons in relation to CO_2 conditions and egg quiescence period.

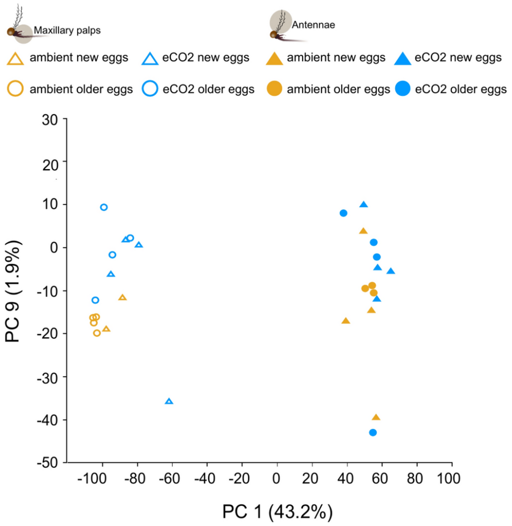

Fig. 1. Elevated CO_2, but not egg quiescence, differentially affects overall gene expression in the peripheral olfactory organs of *Aedes aegypti*. Principal component analysis of antennal and maxillary palp libraries of females emerging from new and older eggs, with short and extended egg quiescent duration, respectively, reared under ambient and elevated CO_2 conditions. A total of 29 libraries were analysed to estimate the change in overall gene expression, in which Principal Component (PC) 1 (43.2%) and PC 9 (1.9%) accounted for the variance between the libraries.

Regulation of peripheral chemosensory genes

Elevation in CO_2 and extended egg quiescence period differentially modulated the expression profile of chemosensory genes, with the highest differential regulation occurring in the maxillary palps.

Odorant receptors

Among the 97 annotated *Ors*, 88 and 3, including *Orco*, were reliably expressed in the antennae and maxillary palps of female *Ae. aegypti,* respectively (Supplementary Table S4). While *Orco* was not significantly regulated, the antennally-expressed *Or50* and *Or86* significantly increased in abundance in females emerging from older eggs, in response to elevated CO_2 conditions (Fig. 4a). The three *Ors* expressed in the maxillary palps were not regulated in response to an elevation in CO_2 or egg quiescence period (Fig. 4b).

Ionotropic receptors

Of the 52 annotated *Irs*, 33 and 4 were reliably expressed in the antennal and maxillary palp libraries, respectively (Supplementary Table S4). The three co-receptors were reliably expressed, with *Ir25a* having a significantly lower abundance in maxillary palps of females originating from eggs that underwent an extended egg quiescence period and then reared under elevated CO_2 conditions (Supplementary Table S4, Fig. 4b). Of the 30 tuning *Irs* expressed in the antennal libraries, *Ir75k* increased in abundance in response to an extended egg quiescence period, when females were reared under ambient CO_2 conditions (Fig. 4b).

Gustatory receptors

Among the 41 annotated *Grs*, 9 and 5 were reliably expressed in the antenna and maxillary palps libraries, respectively (Supplementary Table S4). No *Grs* were differentially regulated in the antennal or maxillary palp libraries (Supplementary Table S4).

Non-canonical chemoreceptor-related families

Of the 14 annotated *Trps*, 7 and 6 were reliably expressed in the antennal and maxillary palp libraries, respectively, none of which were differentially regulated (Supplementary Table S4). Similarly, of the 46 annotated *pickpocket* genes, 15 and 9 were reliably expressed in the antennal and maxillary palp libraries, respectively, none of which showed differential expression in response to elevated CO_2 conditions or egg quiescence period (Supplementary Table S4).

The genes coding for sensory neuron membrane proteins (SNMPs), of which *SNMP1* and *SNMP2* were among the 10 and 11, out of the 13 annotated, reliably expressed *SCRBs* in the antennal and maxillary palp libraries, respectively (Supplementary Table S4). The expression of *SCRB6* and *SNMP1* was downregulated in

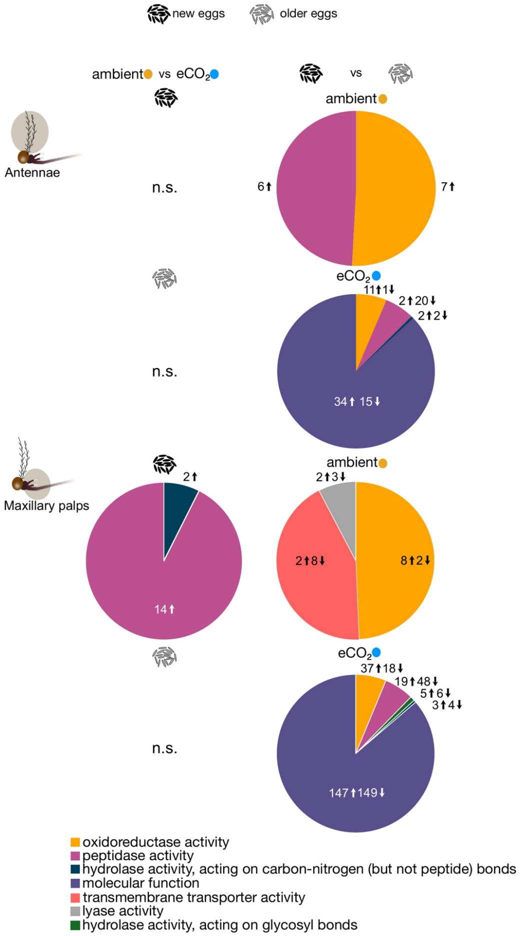

Fig. 2. Gene ontology analysis of differentially expressed genes in the antennae and maxillary palps of *Aedes aegypti*. The olfactory tissues were collected from females reared under ambient and elevated CO_2 conditions, as well as short and extended egg quiescence duration, referred to as new and older eggs, respectively. Pairwise comparisons are arranged in a matrix in response to CO_2 conditions and egg quiescence period. The differentially expressed genes are classified into molecular function ontology, using gene ontology slim categorisation. n.s.: non-significant.

the maxillary palps of females reared under elevated and ambient CO_2 conditions, respectively, in response to extended egg quiescence period (Fig. 4b).

Soluble odorant-binding proteins

The genes encoding for OBPs and CSPs were highly abundant in the antennae and maxillary palps libraries. Out of the 52 annotated *OBPs*, 33 and 35 were reliably expressed in the antennal and maxillary palp libraries, respectively (Supplementary Table S4). Only one *OBP*, *OBP25*, increased in abundance in the antennae of females reared under elevated CO_2 conditions in response to an extended egg quiescence period (Fig. 4a). In the maxillary palp libraries, *OBPs* were differentially regulated in response to elevated CO_2: seven *OBPs* were significantly lower in abundance in females emerging from new eggs, while four *OBPs* were higher in abundance in females emerging from older eggs, in response to elevated CO_2 conditions (Fig. 4b). In response to extended egg quiescence period, the abundance of *OBPs* were differentially regulated in relation to CO_2 condition: seven out of the ten differentially expressed *OBPs* in the maxillary palp libraries of females reared under ambient

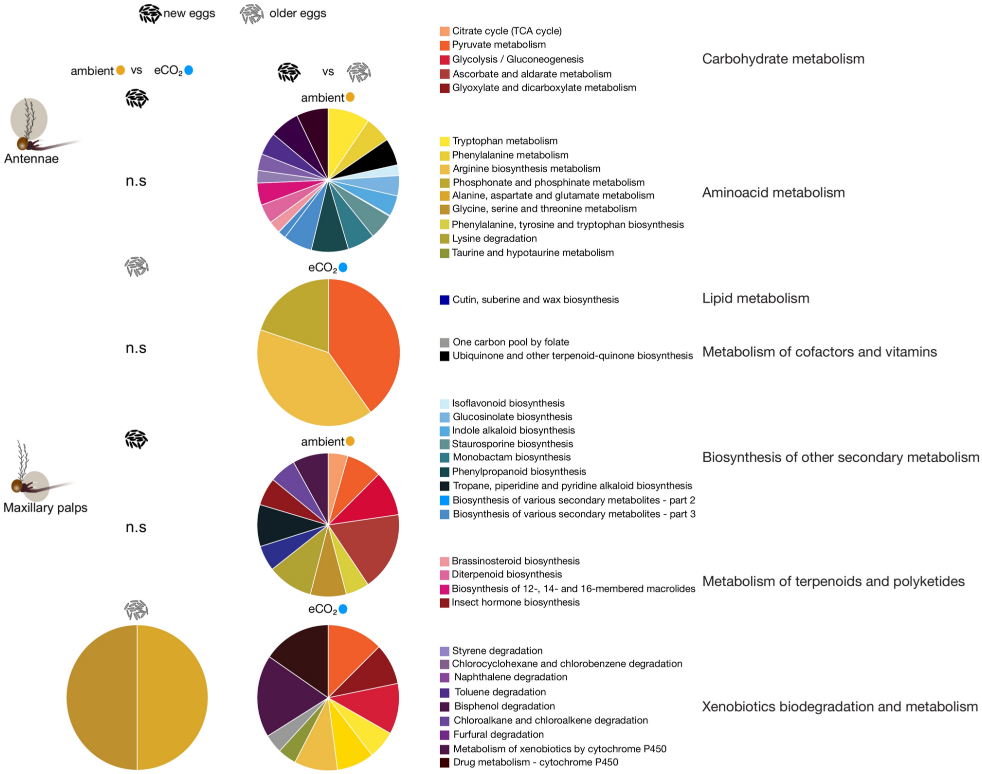

Fig. 3. Kyoto Encyclopedia of Genes and Genomes pathway analysis of differentially expressed genes in the antennae and maxillary palps of *Aedes aegypti*. The olfactory tissues were collected from females reared under ambient and elevated CO_2 conditions, as well as short and extended egg quiescence duration, referred to as new and older eggs, respectively. Pairwise comparisons are arranged in a matrix in relation to the response to CO_2 conditions (eCO_2) and egg quiescence period. The categories are annotated from Vectorbase and further classified into pathways designated by Kyoto Encyclopedia of Genes and Genomes database (https://www.genome.jp/kegg/).

CO_2 conditions were lower in abundance, while nine *OBP*s were higher in abundance in females reared under elevated CO_2 conditions (Fig. 4b).

Out of the 17 annotated *CSP*s, seven and ten were reliably expressed in the antennal and maxillary palp libraries, respectively (Supplementary Table S4). The *CSP*s did not display any differential expression in response to CO_2 conditions and egg quiescence period in the antennal libraries. However, in the maxillary palp libraries, one and three *CSP*s decreased in abundance in females when reared under ambient and elevated CO_2 conditions, respectively, in response to an extended egg quiescence period (Fig. 4b).

Discussion

Based on this transcriptome analysis, the effect of an elevation in CO_2 level, to that predicted under extreme conditions[7], appears to be gene-family specific, while egg quiescent duration has a distinct and overall impact on gene expression, particularly in the maxillary palp. Differential expression of genes in both antennae and maxillary palps involved in metabolism and xenobiotics emphasise a stress response as a consequence of elevated CO_2 and extended egg quiescence duration, similar to the systemic response shown in other insects to environmental stressors[56]. Contrasting regulation of select members of chemosensory gene families, ORs, IRs, SNMPs, OBPs and CSPs, in the antennae and maxillary palp, may regulate the observed differences in resource-seeking behaviour in response to the two external stressors in female *Ae. aegypti*[6]. Overall, this study provides insights into how environmental stress impacts the peripheral olfactory system of insects and ensuing behaviour.

The differential feeding behaviour of *Ae. aegypti* as a result of different egg quiescence durations, and when reared under elevated CO_2[6], while appearing to have no significant generalised effect on gene expression, is

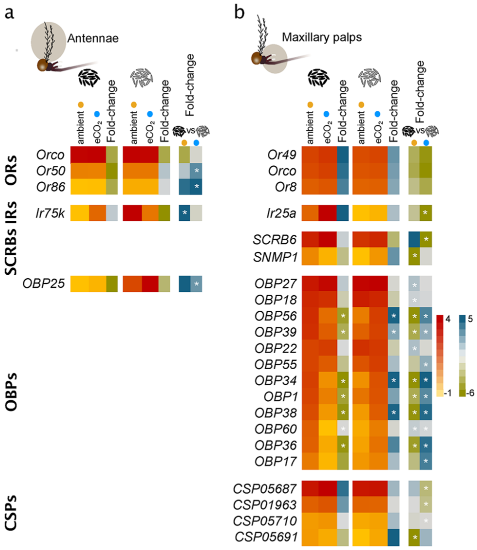

Fig. 4. Differential abundance of chemosensory genes in *Aedes aegypti* in response to elevated CO$_2$ conditions and extended egg quiescence period. The olfactory tissues were collected from females reared under ambient and elevated CO$_2$ conditions, as well as short and extended egg quiescence duration, referred to as new and older eggs, respectively. The abundance of reliably expressed (> 1 transcript per million) chemosensory genes compared between ambient and elevated CO$_2$ (eCO$_2$) levels, as well as egg quiescent periods, in the antennal (**a**) and maxillary palp (**b**) libraries, and demonstrated by fold-change (> 1.5-fold change; FDR > 0.05). Asterisks on fold change denote significant differences between pairwise comparisons.

likely a result of more targeted regulation of genes as indicated in the GO slim and KEGG analyses. The high number of significant DEGs, characterised by GO slim analysis, emphasises an interactive effect of elevated CO$_2$ conditions and extended egg quiescence period on gene regulation in the peripheral olfactory system. The differentially regulated genes, predominantly in the maxillary palp, divides into categories including energy metabolism and xenobiotic response pathways, which is highlighted through KEGG analysis, and emphasises a significant transcriptional regulation of stress-induced genes in an organ that is involved in the detection of CO$_2$ and other host-related chemosensory signals[57,58]. A similar transcriptional regulation of metabolic genes, in response to elevated CO$_2$, has been demonstrated in aquatic invertebrates and insects[59,60]. Tissue-specific effect on gene expression regulation in the olfactory system, in response to elevated CO$_2$, has also been demonstrated in salmon[61]. While elevated CO$_2$ levels do not appear to directly trigger the xenobiotic response pathways, elevated CO$_2$ upregulates the transcription of genes encoding for detoxifying enzymes, including cytochrome P450s[62,63], [this study]. Xenobiotic response genes, including members of the cytochrome P450 family, are regulated in response to a variety of environmental stressors, including volatile compounds[64,65], prolonged exposure to insecticides[66,67], and abiotic stressors[68–70]. Cytochrome P450s act as odorant degrading enzymes in the insect peripheral olfactory system[71]. Hence, the oxidative stress and potential acidification of the sensillum lymph, as a result of the conversion of CO$_2$ into carbonic acid[72], may explain the observed response in this degradation pathway. Acidification of the sensillum lymph influences the folding of OBPs[73–75], which can lead to alterations in protein function. Although mosquitoes acid–base regulate under varying pH conditions[76,77], it remains unclear how the buffering capacity is impacted by prolonged exposure to elevated CO$_2$. Furthermore, how this affects the membrane-bound receptors[78], and the cascading effects on neuronal signalling[79,80], remains to be studied.

Elevated CO$_2$ levels, accentuated by egg quiescence duration, differentially affected the expression of soluble and membrane-bound chemosensory genes, which may directly affect the behaviour of disease-transmitting mosquitoes[6], [this study]. Of the soluble odorant-binding proteins, insect OBPs facilitate odorant transport, odorant-receptor interactions and gain control[81], as well as xenobiotic adaptations[82]. The significant differential regulation of *OBP*s, predominantly in the maxillary palp, emphasises the important role of these genes in response to elevated CO$_2$ levels and extended egg quiescence duration. Of the 12 differentially regulated *OBP*s, only *OBP22* and *OBP39* have been functionally characterised, and demonstrated to detect long-chain fatty acids involved in host- and oviposition-site seeking, respectively[83,84]. The abundance of a subset of *OBP*s, including *OBP56, OBP39, OBP34* and *OBP38*, shifted in response to elevated CO$_2$, from low in new eggs to high in older eggs, suggesting a conserved regulatory pathway for these OBPs in response to stress. Considering the role of

OBPs, the demonstrated regulation of genes will likely affect the interaction between odorant ligands and the membrane-bound receptors.

Among the membrane-bound receptors, the differential regulation of *Or*s in the antenna provides an insight into the regulatory mechanism regulating *Or* expression in response to environmental stress[85], despite the unknown functional relevance of these changes for *Ae. aegypti*[44,86–89]. The absence of regulation in other *Or*s in both antennae[53,90] and maxillary palps[91] suggests that core Or-mediated sensory detection remains largely unaffected, as is the case for other membrane-bound receptors. Among the differentially regulated *Ir*s that have been functionally characterised, the Ir co-receptor *Ir25a* is involved in the detection of amines[92,93], whereas the tuning Ir, *Ir75k*, is sensitive to short-chain carboxylic acids[94]. These chemical classes play important roles in host- and oviposition-site selection[95,96], and the differential regulation of the receptors detecting these odorants may affect the efficient resource seeking by mosquitoes. In *Drosophila*, *Ir25a* is required for context-dependent attraction to CO_2[97], and in female *Ae. aegypti, Ir25a* is co-expressed in the maxillary palp CO_2 sensitive OSN[38]. The functional significance of the lower abundance of *Ir25a* in response to stress requires further investigation. While short-term exposure to elevated CO_2 significantly impact host seeking, as a consequence of sensory constraint[21], the genes encoding for the subunits forming the CO_2 receptor[98] were not regulated in response to developmental exposure to high CO_2. Whether long-term exposure to high CO_2 levels has a similar affect, and how this is regulated is yet unknown. Taken together, the interaction of elevated CO_2 and extended egg quiescence differentially affect the expression of chemosensory genes that likely play key roles in regulating mosquito behaviours.

This study provides evidence that anthropogenic climate change factors, such as elevated CO_2, interact with other stress factors, such as egg quiescence duration, elicit a stress response in the peripheral olfactory system of mosquitoes and that the capacity of females to detect ecologically-relevant volatile organic compounds may be hampered. While previous studies have demonstrated negative effects of elevated CO_2 and egg quiescence duration on life-history parameters of both aquatic and terrestrial stages of *Ae. aegypti,* and subsequent carry-over effect on the feeding response of adult females, future experiments are required to assess how these stress factors affect odour-mediated behaviour and physiology.

Methods

Mosquito rearing and tissue collection

For general colony maintenance, *Ae. aegypti* (Rockefeller) were maintained under 27 ± 2 °C, 65 ± 5% relative humidity and a 12 h: 12 h light: dark cycle. The adults had ad libitum access to 10% sucrose (w/v). Females were blood fed with defibrinated sheep blood (Håtunalab AB, Bro, Sweden), using a membrane feeding system (Hemotek Ltd, Blackburn, UK) for egg production. The eggs, deposited on moist filter paper, were collected, dried, labelled and stored for subsequent experiments to account for different egg quiescent periods. The CO_2 acclimatization experiments were conducted in two high-precision climate chambers, in which temperature, humidity and light conditions were maintained as above. The CO_2 concentration in the chambers was 400 ppm (current ambient), and 1000 ppm (elevated CO_2), respectively, in which pure CO_2 (Strandmöllen, Ljungby, Sweden) was delivered and mixed into the ventilation system. Age-controlled eggs (2-week or 3–6-month quiescent periods) were introduced to each experimental chamber, in which eggs from the same cohort were divided equally between two chambers, resulting in a larval density of 100 larvae per 600 ml of water, in each rearing tray. The larvae were fed with fish food (TetraMin® Flakes, Melle, Germany) daily (1 mg larvae^{-1}), normalised for larval mortality. Upon pupation, individual pupae were collected into small (30 ml) plastic cups with distilled water and placed into Bugdorm cages (17.5 cm × 17.5 cm × 17.5 cm; Megaview Science Co., Ltd, Taichung, Taiwan). The emerging adults had ad libitum *acce*ss to water until tissue was collected.

Collection of teneral (30 ± 6 h) female antennae and maxillary palp were done at Zeitgeber time 10–12, *i.e.*, the peak diel activity period of *Ae. aegypti*[99]. For the dissection, females were anesthetised on ice, and the tissues removed using a pair of fine sterilised forceps, with separate pairs of forceps used for each olfactory tissue type, and then placed into 200 µl of RNAlater (Thermo Fisher Scientific, Gothenburg, Sweden). Forceps were sterilised in between each biological replicate using 70% ethanol. The tissue was stored at room temperature overnight, then at -20 °C overnight, and thereafter at -80 °C until RNA extraction. A total of 16 antennal libraries were generated, with each library comprising pooled tissues from 50 individuals per replicate from different cohorts, across two CO_2 conditions, two egg quiescence periods, and four biological replicates (50 tissues × 2 CO_2 levels × 2 quiescent periods × 4 replicates = 800 pairs of tissues). Similarly, 16 maxillary palp libraries were constructed using the same pooling strategy, yielding an additional 800 pairs of tissues. In total, 1,600 pairs of tissues were collected for all (32) libraries.

RNA extraction and sequencing

Total RNA extraction was performed using the RNeasy microRNA kit (Qiagen, Hilden, Germany) following the manufacturer's protocol with an additional step of quick freezing with liquid nitrogen to facilitate the homogenisation of the tissues. The RNA extracted was immediately stored at -80 °C and later quantified using the TapeStation system 4150 (Agilent Technologies, Inc, Santa Clara, US). The samples were shipped on dry ice to Eurofins Genomics (Constance, Germany), where INVIEW ultra-low transcriptome libraries were constructed using NovaSeq Illumina genome sequencing technology (Illumina NovaSeq 6000 S4 PE150 XP). The cDNA library construction was realised using Eurofins proprietary protocol, generating 2 × 150 bp coverage paired-end reads with a depth of 20 million paired-end reads (Supplementary Table S5).

Read mapping and annotation

Prior to the quantitative assessment of the library sequences, the samples underwent quality control steps involving the removal of adapter sequences, and discarding sequences with a Phred score of below 40, using CLC

Genomics Workbench (23.0.5, Qiagen, Aarhus, Denmark). Three libraries were removed from further analysis due to cross-contamination between tissues (Supplementary Table 1)[100]. The sequences were mapped to the *Ae. aegypti* reference genome (VectorBase: *Aedes aegypti* LVP_AGWG, AaegL5.3).

PCA analysis

Principal component analysis (PCA) was performed to estimate the effect of elevated CO_2 and egg quiescence period on the overall expression profile. The high-dimensional dataset containing the antennal and maxillary palp libraries was projected onto two-dimensional components to determine the variance between libraries using the toolbox for RNA-seq data in CLC Genomics Workbench. The individual and interactive effect of CO_2 level and egg quiescent period on each olfactory tissue was assessed through permutational multivariate analysis of variance (PERMOVA) using "adonis2" function under the *vegan* package in RStudio.

RNA seq and differential expression analysis

For the transcriptome analysis, transcripts per million (TPM) was used, with a reliable expression of genes determined to be above a threshold of 1 TPM. Differential transcript abundance was analysed using a negative binomial distribution with a gamma-Poisson mixed distribution in CLC Genomics Workbench (https://digitalinsights.qiagen.com/). To account for false positives during the statistical tests, the false discovery rate (FDR) with p-value correction was performed using the Benjamin-Hochberg method[101]. The analysis generated a mean abundance value, fold change (FC) and FDR p-values that were accessed for differential expression. Genes were considered significantly differentially expressed when fold change > 1.5 and FDR p-value < 0.05.

Functional enrichment analyses

To assess the effects of elevated CO_2 and extended egg quiescence period on molecular function level and metabolic pathways, a gene ontology (GO) analysis and KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis were performed. The GO and KEGG terms used for the identified differentially expressed genes (DEGs) in the antennae and maxillary palps, were identified from VectorBase (AaegL5.3, Release 68). The VectorBase GO enrichment tool was used for assessing the molecular function, with both computed and curated evidence limited to GO slim terms. The VectorBase metabolic pathway enrichment was used for KEGG analysis. The significance cut-off was set to alpha $(\alpha) = 0.05$.

Data availability

All data generated are presented in the publication. The transcriptome data generated and analysed during this study is available in the NCBI project database, with BioProject ID: PRJNA1195965. https://www.ncbi.nlm.nih.gov/sra/PRJNA1195965.

Received: 10 December 2024; Accepted: 9 April 2025

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Acknowledgements

We acknowledge the help provided by the Biotron maintenance staff at SLU.

Author contributions

SN and RI conceived the idea, SN, SRH and RI designed the methodology and SN collected and analysed the data. SN drafted the manuscript, and RI critically revised the manuscript. All authors gave final approval for publication.

Funding

Open access funding provided by Swedish University of Agricultural Sciences.

Max Planck Center next Generation Insect Chemical Ecology.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-98159-w.

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ACTA UNIVERSITATIS AGRICULTURAE SUECIAE
DOCTORAL THESIS NO. 2025:45

Internal and external factors shape mosquito physiology and behaviour. This thesis explores how predicted climatic changes and female physiological state modulate the life history parameters, molecular biology and neuroethology of *Aedes aegypti.*

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ISSN 1652-6880
ISBN (print version) 978-91-8046-480-2
ISBN (electronic version) 978-91-8046-530-4