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The evolutionary ecology of vector-parasite interactions

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Abstract

Ecological interactions between parasites and their within-host environment and within-vector environment (in the case of vector-borne diseases) shape the severity and transmission of infections. Therefore, applying evolutionary ecology frameworks to explain how and why parasites respond to environmental variation is becoming increasingly more common. A greater understanding of within-host and within-vector ecology can improve predictions of parasite evolution in response to disease control interventions and other environmental changes. Malaria is the deadliest vector-borne disease and is caused by parasites of the *Plasmodium* genus which are transmitted between vertebrate hosts via mosquitoes (often *Anopheles* spp.). However, despite its public health importance and decades of intensive research, fundamental aspects of malaria transmission remain poorly understood. In particular, the sources of variation in parasite life history traits that shape within-vector development and onward transmission to a new vertebrate host have received little attention.

By integrating evolutionary ecology, parasitology and vector biology, I investigate how environmentally determined variation in vector traits shapes parasite traits expressed within the vector, and the subsequent implications for between-host transmission and parasite evolutionary potential. It is becoming increasingly important to understand how parasites evolve to cope with the challenges and/or exploit the opportunities provided by their within-vector environment, because vector control tools are altering vector genotypes and phenotypes. Therefore, I firstly created a framework using evolutionary ecology to predict how parasites could evolve in response to vector control tool-imposed changes in their vectors, collating the consequences of these changes into overarching agents of selection acting on parasites (Chapter 2). Following this, I focussed on the following key aspects that arose, examining: (i) whether genetic variation within parasite populations influences the potential for selection to act on fitness-related traits (Chapter 3), (ii) the extent of plasticity for parasite traits that are altered in response to novel within-vector environments (Chapter 4), (iii) how changes to vector biology as a consequence of vector control directly and/or indirectly affect parasite fitness (Chapter 5), and (iv) if parasite transmission potential and fitness can be better estimated (Chapter 6).

My empirical results reveal that there are strain-specific differences in within-vector parasite traits important for transmission (Chapter 3), suggesting that there is underlying genetic variation for natural selection to act upon. In addition, I show that both parasite (Chapter 4) and vector traits (Chapter 5) exhibit plastic strategies in

response to variation in the availability of nutritional resources. My findings (Chapter 4) suggest that while parasites are constrained by limited access to nutritional resources, they will actively adjust their within-vector development to best exploit the resources available in ways that maximise transmission. I also demonstrate (Chapter 5) that the timing and propensity of vector biting is dependent on their nutritional state; while parasites produce fewer progeny in starved mosquitoes, these mosquitoes have a higher propensity to bite, and are also more likely to forage at a non-classical biting time when humans are unprotected by bed nets. Finally, I develop and test a non-destructive assay to track parasite development over time (Chapter 6) and demonstrate that while parasites expelled in mosquito saliva during sugar feeding are detectable, increasing the detection rate is required to maximise the utility of these assays for quantifying transmission dynamics.

Together, my results reveal that malaria parasites demonstrate considerable variation in within-vector traits and can plastically respond to changes in their environment in manners that appear adaptive. Thus, my findings suggest that parasites have the ability to evolve and plastically change fitness-determining traits, many of which are affected by vector control tool use. However, my work also demonstrates that within-vector environmental variation (such as resource availability) can influence the transmission potential of parasites and vectors in opposing ways and may have complex consequences for disease dynamics. Therefore, investigating how and why both vector and parasite phenotypes respond to environmental change is necessary for understanding for how natural selection will shape disease transmission and parasite evolution, especially in response to current and future control interventions.

Lay summary

Parasites need to transmit between hosts to reproduce. For some parasites, an insect vector is required to move parasites between hosts, with certain parasite species undergoing a period of development within the insect. The interactions between parasites and their environment, both inside their host and insect, determines how much infections spread and how severe their symptoms are to hosts. Parasites, particularly those that cause malaria, are well known to adjust their behaviours in response to different circumstances that they face during infections. Understanding how parasite responses to situations such as their insect vector having a short lifespan, limited food reserves for the parasite to exploit, or biting hosts to acquire blood at different times of day, is important for predicting how parasites might evolve in response to disease control efforts and other environmental changes. The deadliest disease spread by insects is caused by malaria parasites, which are transmitted between hosts by mosquito bite. Despite the global impacts of malaria on health and economies, how malaria parasites alter their behaviours to improve their chances of transmitting between hosts is poorly understood. My thesis investigates the different strategies that malaria parasites use to survive in the mosquito and transmit between hosts, and how this influences disease transmission and parasite evolution. It is becoming increasingly important to understand how parasites cope with and/or exploit opportunities that arise within the mosquito, because tools used to control mosquitoes have led to changes in mosquito biology.

In Chapter 2, I developed an evolutionary ecology framework to predict the strategies parasites might adopt in mosquitoes altered by the use of control tools such as insecticide treated bed nets. This framework highlighted that more research was required on many fundamental aspects of parasite and mosquito interactions to be able to predict parasite responses, so I then addressed some of those aspects. In Chapter 3, I show that genetically distinct malaria parasite strains adopt different strategies for exploiting mosquitoes, suggesting that parasites optimise transmission in different ways. In Chapter 4, I demonstrate that parasites adjust their behaviour in response to the amount of food resources available within mosquitoes, again in ways that are likely to maximise their chance of transmission. In Chapter 5, I reveal that altering mosquito nutritional status (by altering food availability) shifts the time of day that mosquitoes bite, which affects the likelihood of mosquitoes coming into contact with hosts and thus transmitting parasites. Finally, in Chapter 6 I develop a new method to track parasite development in the mosquito over time. Overall, my results suggest that malaria parasites can alter their behaviours and have the potential to adapt to changes in their within-mosquito environment. However, my findings also highlight that the factors I have studied can have opposing impacts on parasites and mosquitoes, which affects the likelihood of transmission between hosts in unexpected ways.

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Signed Declaration

I declare that this thesis has been composed by me under the supervision of Professor Sarah Reece and has not been submitted, in whole or in part, for any other degree or professional qualification. I use the word 'we' throughout the data chapters because I have written them as papers. My contribution to each chapter and that of other authors have been explicitly listed below:

Chapter 2: This chapter is published in *Trends in Parasitology*. I researched and wrote the manuscript. Sarah Reece advised the writing and contributed ideas. Vicki Ingham and Craig Walling helped with interpretation of the insecticide-resistance literature and life history concepts, respectively.

Chapter 3: Ronnie Mooney and Aidan O'Donnell assisted with data collection. I designed the experiment, analysed the data and wrote the manuscript, advised by Sarah Reece.

Chapter 4: This chapter is published in *Frontiers in Malaria*. The experiments were designed by me with help from Sarah Reece, Aidan O'Donnell and Petra Schneider. Aidan, Petra, Ronnie Mooney and honours student Gregor Doyle assisted with data collection. Petra guided me through the molecular work. I analysed the data and wrote the manuscript, advised by Sarah.

Chapter 5: This chapter was the product of a collaboration between Yaw Afrane, Samuel Rund, and Maxwell Machani. I designed the semi-field experiments, with help from Yaw, Sam and Max. Rahim Mohammed, Sam and Max assisted with data collection for the semi-field experiments and field collections. I analysed the data and wrote the manuscript, advised by Sarah Reece.

Chapter 6: This chapter is published in *Parasites & Vectors*. I designed and conducted the experiments, and wrote the manuscript, advised by Petra Schneider and Sarah Reece. I analysed the data under the guidance of Petra.

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List of Figures, Tables and Boxes

Chapter 1. Introduction

1.1 The evolutionary ecology of infection

Understanding how natural selection shapes the traits of infectious disease-causing organisms is central to predicting their severity and spread, and the efficacy of interventions. While research has historically focussed on the molecular and cellular mechanisms governing host-parasite interactions, an evolutionary ecology perspective is increasingly being applied (Leggett et al., 2014; Schneider and Reece, 2021; King et al., 2023; Natterson-Horowitz et al., 2023). Testing hypotheses developed using evolutionary and ecological principles has revealed important insights into the biology of parasites, including surprisingly sophisticated strategies that are deployed to cope with the challenges of a parasitic lifestyle, including drug treatment (Reece et al., 2010; Schneider et al., 2012b, 2018a; Birget et al., 2018), and have helped to explain why variation exists for traits such as virulence (De Roode et al., 2005; Bell et al., 2006; Schneider et al., 2012b). Conversely, the varied lifestyles and lifecycles of parasites provides a novel testing ground for the generality of evolutionary theories originally developed to explain the lifestyles of free living animals (West et al., 2006; Reece et al., 2008).

Interactions between parasites, hosts and vectors (in the case of vector-borne parasites) span all scales of biological organisation, from cellular and molecular processes, to the phenotypes and behaviours of all parties, to population-level processes. Thus, a full understanding of infections and how they evolve requires phenomena to be integrated across these scales (Schneider and Reece, 2021). When applied to infections, evolutionary ecology aims to understand how and why interactions between parasites and the environments they experience during infections shape the evolution of parasite phenotypes (i.e. traits, behaviours, strategies) underlying within-host survival and between-host transmission (Paul et al., 2003; Reece et al., 2009; Mideo and Reece, 2012; Neal and Schall, 2014a; Greischar et al., 2016; Schneider and Reece, 2021). Explaining when, and why, parasites adopt certain phenotypes bridges the intersection between cellular/molecular and population processes. For example, uncovering a novel parasite strategy opens up the search for the underlying mechanisms and allows its epidemiological consequences to be predicted (Schneider and Reece, 2021; Natterson-Horowitz et al., 2023). Combining mechanistic, ecological and evolutionary insight can reveal new

targets for infection control interventions and how to make them robust to parasite counter-evolution.

Understanding parasites strategies is challenging because infections are highly dynamic processes, presenting parasites with variable environments during infections within individual hosts/vectors and also in the conditions encountered in different hosts/vectors. Environmental factors that shape parasite fitness (i.e. survival and onward transmission) include the amount and age of red blood cells in the host's blood, and nutritional resources, immune responses, and the presence of co-infecting parasites in both the host and vector (Bell et al., 2006; Reece et al., 2009; Babayan et al., 2010; Pollitt et al., 2011b; Birget et al., 2017; Simões et al., 2017) (see Chapters 4 and 5). Thus, parasite traits can be influenced by multiple interacting factors, and the ways that parasites respond can also generate variation in the within-host/vector environment (Schmid-Hempel, 2021). For example, malaria parasites sexually reproduce, and within the vertebrate host must divide resources between ensuring survival of the infection via asexual replication in the blood and investment in sexual reproduction via production of non-replicating gametocytes. The proportion of asexual parasites that commit to producing gametocytes is termed the ‘conversion rate’, which can be adjusted in response to within-host conditions. Under mild stress (e.g. low dose drug treatment), parasites adopt ‘reproductive restraint’, where they increase investment in asexual replication at the expense of short term transmission to improve prospects for within-host survival and future transmission (Reece et al., 2010; Carter et al., 2013; Birget et al., 2017, 2018; Schneider et al., 2018a; Portugaliza et al., 2020). Thus, being able to plastically adjust their strategy throughout the course of an infection in response to changing conditions (i.e. phenotypic plasticity) allows parasites to maximise their within-host fitness.

While much progress has been made in explaining the within-host ecology of parasites and the strategies they have evolved, for vector-borne diseases like malaria, within-vector ecology is very poorly understood. However, there are lots of analogous sources of variation that should shape within-vector development and onward transmission (Lefevre et al., 2017). Within-vector parasite ecology has likely been overlooked because it is not as relevant to clinical disease and is often more challenging to study than infections in a mammalian host. However, parasite fitness is dependent on between-host transmission, and so understanding the within-vector ecology of parasites is necessary for explaining and predicting the spread of disease. In particular, for malaria, insecticide-based interventions which target vectors (e.g. insecticide treated bed nets) have historically been the most successful at curbing

disease (Bhatt et al., 2015; Oke et al., 2022), but progress has stalled in the past few years (Noor and Alonso, 2022), largely due to the evolution of insecticide resistance. Thus, understanding how parasite traits are shaped by vector responses to vector control tools can inform approaches for disease control and prevent unfavourable parasite counter-evolution.

In this thesis, I demonstrate how evolutionary ecology concepts can be applied to explain within-vector parasite strategies for survival and reproduction across their life cycle. My research focuses on malaria parasites because of their public health importance and because they are a tractable model system, thanks to the wealth of tools available and depth of understanding of their biology from decades of research into their natural history, immunology, genomics, and molecular biology, though I expect the general findings to apply to other vector-borne parasites. Throughout this thesis, I interchangeably refer to traits, phenotypes, behaviours and strategies exhibited by parasites, and denote all agents of infection (e.g. parasites, pathogens, microbes) as parasites. In the following sections I provide primers covering core evolutionary ecology concepts, transmission traits of malaria parasites, and the extent of variation that exists in both parasite traits and the within-vector environments that parasites experience. I then introduce the focus of my thesis and highlight that understanding within-vector strategies is particularly important, given that their vectors are currently evolving in response to the use of vector control tools.

1.2 Life history theory and phenotypic plasticity

The life history of an organism encompasses its schedule of growth, reproduction and survival (Neal and Schall, 2014a). Life history theory aims to explain how natural selection maximises an organism's survival and reproductive success (i.e. fitness) through the expression of a combination of advantageous traits (Roff, 1992; Stearns, 1992). However, life history evolution is an optimality problem, because organisms must divide finite resources between competing traits, such as growth versus reproduction (Stearns, 2000). Thus, the possible combination of traits that can be expressed is limited by trade-offs and constraints (Stearns, 1989), not just between fitness components (i.e. growth versus reproduction), but also within fitness components (e.g. reproduction). For example, an organism could invest in a high number of low quality offspring, or a smaller number of higher quality offspring (Stott et al., 2024). Natural selection should optimise the allocation of resources in such a way to optimise an organism's fitness, with different ecologies and lifestyles selecting

for different strategies (Stott et al., 2024). Defining what constitutes an organism is clear for metazoans such as birds or mammals. When applied to single-celled parasites, clonally-related parasites within an infection (i.e. those belonging to the same genotype) are analogous to a colony of social insects, where the parasite genotype represents the 'organism' and is the comparable target of natural selection (West et al., 2006; West and Kiers, 2009). Thus, for malaria parasites, within-host asexual replication represents growth and survival, production of gametocytes represent reproductive effort (Schneider and Reece, 2021), and within-vector development represents the production of offspring.

Like all organisms, parasites do not evolve in constant environmental conditions, generating variation in the optimal life history strategies which maximise their fitness. For example, throughout an infection, the relative importance of investing in survival (i.e. asexual replication) versus transmission can change due to variation in red blood cell availability (Schneider et al., 2018a). Thus, the environment shapes selection on parasite traits, and influences the trade-offs occurring between traits (Reece et al., 2009). Traits can be genetically 'fixed' strategies, where each genotype produces a different phenotype, regardless of environmental conditions. Assuming sufficient genetic variation for the trait exists, natural selection will favour the genotypes which produce the phenotype most fitted to the average, or most frequently encountered, environmental condition. In contrast, traits can exhibit phenotypic plasticity, defined as a single genotype producing different phenotypes dependent on environmental conditions (Pfennig, 2021). Plasticity is adaptive if a phenotypic alteration in response to different environmental conditions maintains or increases fitness (Pigliucci, 2005; Ghalambor et al., 2007). For example, bacteriophages can plastically adjust their lysis time in response to competition with unrelated phages inside bacterial host cells, maximising fitness in both single and co-infections (Leggett et al., 2013). However, it is often difficult to differentiate between adaptive or non-adaptive plasticity, where parasite traits are either actively altered, or changed via a constraint being imposed or released outside of the parasite's control (Schneider and Reece, 2021).

Compared to fixed responses, which evolve via selection on genes across generations (i.e. microevolution), plastic responses can occur within a generation (e.g. via epigenetic modifications), allowing organisms to cope with environmental variation on a short time frame (Whitman and Agrawal, 2009; Pfennig, 2021). However, phenotypic plasticity is expected to be constrained by costs and limits. Costs reflect factors that reduce the fitness of plastic organisms, such as developmental machinery required to produce a variety of phenotypes or sensing

mechanisms required to detect environmental changes (Scheiner, 1993). Limits reflect the inability of a plastic organism to produce the optimal phenotypes under all differing environmental conditions (Scheiner and Holt, 2012; Murren et al., 2015). Thus, plasticity is only expected to evolve when environmental conditions are variable but predictable, environmental state is reliably associated with cues, and there are low costs (Scheiner, 2020). In addition, suitable genetic variation for plastic responses must exist (Pfennig, 2021). In contrast, if environments are variable but unpredictable or poorly associated with cues, a 'bet-hedging' strategy may evolve. Bet-hedging involves a genotype producing multiple diverse phenotypes suited to different environmental conditions, with the assumption that some forms will be adaptive (Viney and Reece, 2013). While reducing short-term (arithmetic) fitness, this strategy can maximise (geometric) fitness in the long run by preventing extinction. For example, *var* gene switching in malaria parasites is an immune evasion strategy assumed to be bet-hedging (Llorà-Batlle et al., 2019). However, other parasite strategies such as adjusting conversion rate in response to environmental conditions are more likely to be adaptive plastic responses, because they are adjusted in repeatable and directional manners (Schneider and Reece, 2021).

The evolution of fixed traits and phenotypic plasticity are not mutually exclusive (Lefevre et al., 2017). Plastic responses can constrain evolution in response to environmental change by allowing organisms to adopt the fitness-maximising phenotype via plasticity alone. Alternatively, plasticity can facilitate evolution by buffering populations from extinction under novel conditions, allowing more time for beneficial mutations to arise (Chevin et al., 2010; Fox et al., 2019). In addition, because genotypes can differ in their response to environmental change (genotype-by-environment interactions, GxE) (Pigliucci, 2005), environmental change can expose previously cryptic genetic variation (Ghalambor et al., 2007; Paaby and Rockman, 2014) to natural selection, facilitating evolution. Understanding when plasticity in parasite genotypes will facilitate or constrain evolutionary change has important implications for disease control (Carter et al., 2013; Oke et al., 2022). For example, adopting reproductive restraint in response to low drug doses relaxes the strength of selection for antimalarial resistance, but it also increases parasite asexual density in the blood, maximising the number of genomes and thus the chance for *de novo* resistance mutations to occur (Schneider and Reece, 2021).

1.3 *Plasmodium* life cycle and transmission traits

Malaria parasites (*Plasmodium* spp.) are the causative agents of malaria, and can infect a variety of vertebrate hosts, including humans (e.g. *P. falciparum*, *P. vivax*), rodents (e.g. *P. chabaudi*, *P. berghei*), lizards (e.g. *P. mexicanum*) (Schall, 2011) and birds (e.g. *P. gallinaceum*) (Rivero and Gandon, 2018). Despite their diversity, the life cycle follows the same basic steps, with several rounds of asexual replication occurring in a vertebrate host, followed by sexual reproduction within a dipteran vector (Pacheco and Escalante, 2023) (Figure 1.1). An infected vector (e.g. female *Anopheles* spp. mosquitoes for human and rodent malarias) injects *Plasmodium* sporozoites into the skin of the vertebrate host (Hopp et al., 2015, 2021). From here, sporozoites enter the bloodstream and start the exoerythrocytic phase by invading a specific tissue (e.g. the liver in human and rodent malarias). During this stage, sporozoites multiply and differentiate into merozoites, and after several rounds of replication, thousands of merozoites egress from the host tissue and enter the blood stream.

Asexual replication is initiated when merozoites enter red blood cells (RBCs), progressing through a number of developmental stages (ring stage, trophozoite, schizont) culminating in replication to produce progeny (termed merozoites) which egress from the RBC. Merozoites invade uninfected RBCs to initiate a new cycle of asexual replication, termed the intraerythrocytic development cycle (IDC). Every IDC, a small proportion (0.01-1%) of asexual parasites commit to developing into non-replicating sexual stage gametocytes (termed conversion) (Carter et al., 2013; Dogga et al., 2024). Gametocytes are the parasite form responsible for transmission to vectors; while both asexual stages and gametocytes are taken up when a vector takes a blood meal from an infected host, asexuals die while gametocytes begin “sporogony” (Mideo et al., 2013).

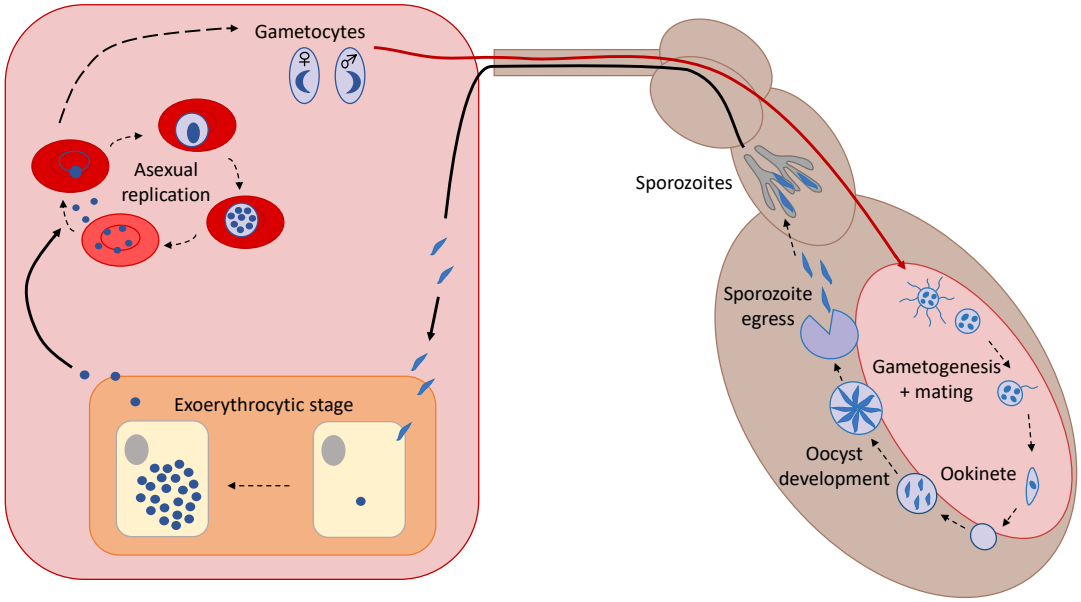

Figure 1.1. The lifecycle of *Plasmodium* parasites. The left panel depicts the within-host stages of the life cycle, including the initial exoerythrocytic replication, followed by cycles of asexual replication in the blood. Every cycle, a small proportion of asexual parasites commit to becoming sexual stage gametocytes, which are taken up by a vector (right panel) during a blood meal. Parasites undergo sporogony in the vector for approximately 10-20 days, producing infectious sporozoites which migrate to and reside in the salivary glands. Sporozoites are injected into the blood stream of a new vertebrate host whilst the vector blood feeds, then they migrate to a specific tissue for their exoerythrocytic stage and the life cycle begins again.

Within the vector, parasites undergo sporogony, which involves several developmental stages over the course of approximately 10-20 days (Ohm et al., 2018; Childs and Prosper, 2020). Gametocytes rapidly differentiate into haploid male and female gametes, which mate and fuse to become a diploid zygote (Baton and Ranford-Cartwright, 2005; Talman et al., 2014). Zygotes transform into motile ookinetes, which traverse the midgut wall approximately 18-30 hours after blood feeding to form oocysts. The oocyst stage is the longest developmental stage of sporogony, lasting between 1-2 weeks depending on the *Plasmodium* species (Smith and Barillas-Mury, 2016). Within oocysts, parasites replicate asexually to form hundreds to thousands of sporozoites, which egress into the vector haemolymph and migrate to the salivary glands (Frischknecht and Matuschewski, 2017). After invasion of the salivary glands, sporozoites change their infective and metabolic state (Bogale

et al., 2021), and are injected into a new host during a subsequent blood meal. Sporozoites can survive in the glands for several weeks, but their infectivity, motility and density reduce over time (Porter et al., 1954; Van Schuijlenburg et al., 2024). The length of time it takes for sporogony to complete and a mosquito to become infectious to a vertebrate host is termed the extrinsic incubation period (EIP), and is typically measured as the time at which sporozoites are observed in the salivary glands (Ohm et al., 2018). However, whether this is the most appropriate measure for the EIP and subsequent infectivity of mosquitoes to vertebrate hosts remains unclear (see Chapter 6).

1.4 Variation in transmission traits

While the basic features of the life cycle are conserved, malaria parasites exhibit extensive variation in traits associated with growth and survival (Table 1.1). Within the host, the length of the exoerythrocytic stage varies across species (Stephens et al., 2012), as does the length of the IDC within the blood (Mideo et al., 2013). In addition, asexual replication can be synchronous (e.g. *P. chabaudi*, *P. falciparum*), or asynchronous (e.g. *P. berghei*) (Greischar et al., 2019, 2024; O'Donnell and Reece, 2021), the number of merozoites produced per infected RBC ('burst size') can range from 4 to 90 dependent on species (Schall, 1990; Kissinger et al., 2002; Reilly et al., 2007; Birget et al., 2019), and species show different preferences for infecting young (reticulocytes) and mature RBCs (Cromer et al., 2006; Lim et al., 2016; Leong et al., 2021, 2022). Within species, and throughout infections, malaria parasites also vary in asexual cycle characteristics; for example, *P. chabaudi* genotypes have different cell cycle durations and respond differently to changes in RBC age structure (Birget et al., 2019). Reproductive traits in the host, which affect between-host transmission, also exhibit variation. For example, the sex ratio of male to female gametocytes, and the conversion rate, show marked differences across species, within species, and throughout the course of infections (Pollitt et al., 2011b; Carter et al., 2013; Portugaliza et al., 2020; Schneider and Reece, 2021; Stewart et al., 2022).

Life history theory has been used to explain variation in within-host traits, including both asexual and reproductive traits (Table 1.1). For example, an evolutionary ecology framework can be applied to explain synchrony in the IDC observed across multiple malaria parasite species, like *P. falciparum* and *P. chabaudi*. Synchrony appears costly, because it intensifies competition between offspring, but there are potential benefits. For example, pulses in parasite numbers may overwhelm host

defences, analogous to organisms using synchronised reproduction to satiate predators (Greischar et al., 2014). In addition, parasites exploit a rhythmic resource, isoleucine, which arrives in the blood in a periodic manner because of the host's feeding-fasting schedule (Prior et al., 2021). This may explain why having a synchronous IDC coordinated to a host's circadian rhythm matters for parasite fitness (O'Donnell et al., 2011). Parasites can also respond to within-host cues to plastically adjust their investment in reproductive effort (conversion) to maximise fitness (Carter et al., 2014). For example, if a parasite genotype invests too heavily in reproductive effort, it may not produce enough asexual parasites to maintain its infection, reducing the transmission window. Alternatively, investing heavily in asexual replication could curtail transmission efforts if infections are too virulent and the host dies prior to transmission occurring (Mideo and Reece, 2012). Parasites balance these scenarios by adopting reproductive restraint during periods of minor stress to ensure within-host survival and future transmission (Carter et al., 2013; Birget et al., 2017; Schneider et al., 2018a), but in extreme scenarios where host death is likely or the infection will be cleared, they increase conversion as a last ditch attempt to ensure transmission to a vector at the expense of within-host survival (i.e. 'terminal investment') (Buckling et al., 1997; Peatey et al., 2009; Schneider et al., 2018a). For example, under high doses of drugs where parasite death rate exceeds the capacity for proliferation, malaria parasites increase investment in conversion (Schneider et al., 2018a).

In contrast, variation in within-vector traits is observed but has received significantly less attention than within-host traits (Lefevre et al., 2017) (Table 1.1). For example, the EIP varies within and across *Plasmodium* species (Fialho and Schall, 1995; Ohm et al., 2018; Childs and Prosper, 2020; Guissou et al., 2023). Furthermore, oocyst burden, oocyst productivity (i.e. sporozoites per oocyst) and sporozoite burden differ across species (Ferguson et al., 2003a; Spence et al., 2012; Pollitt et al., 2013; Bompard et al., 2020; Kanatani et al., 2024). For example, the rodent malaria *P. yoelii* produces fewer sporozoites per oocyst compared to the human malaria *P. falciparum* (Kanatani et al., 2024). Within species, *P. chabaudi* genotypes and *P. falciparum* isolates vary in their infectivity to different mosquito species (Ferguson et al., 2003a; Molina-Cruz et al., 2015), and *P. chabaudi* genotypes differ in the number of oocysts per midgut (Ferguson et al., 2003a) (see Chapter 3). For *P. chabaudi*, it has been suggested that more virulent genotypes are more infective to *An. stephensi* because they produce higher gametocyte densities (Mackinnon and Read, 1999a; Ferguson et al., 2003a). It is surprising that the sources of variation in within-vector traits have been overlooked, given that parasites rely on their within-vector environment for

development and subsequent onward transmission, exploiting the vector's resources for growth and evading its immune system (Shaw et al., 2022) (see Chapter 4).

In addition, parasites experience extensive variation in their within-vector environment (Cator et al., 2020). For example, mosquito vectors exhibit variation in the resources they can acquire and when this occurs (Rund et al., 2016; Shaw et al., 2022) (see Chapters 4 and 5). This influences the resources available for parasites to exploit, and can also cause variation in other factors such immune responses (Rund et al., 2011; Murdock et al., 2013). For example, *An. stephensi* mosquitoes are more susceptible to *P. chabaudi* infection in the day time (Schneider et al., 2018b), likely because immune responses are weaker during the mosquito's rest phase. Other environmental factors, including larval nutrition, abiotic temperature, co-infection, insecticide resistance status and insecticide exposure, can also influence oocyst prevalence and density (Alout et al., 2014a; Pollitt et al., 2015; Shapiro et al., 2016, 2017; Suh et al., 2020; Adams et al., 2023), but few studies have investigated sporozoite infectivity and densities, which are necessary for onward transmission. Development in the vector can also have knock-on consequences for within-host dynamics (see Chapter 3). For example, mosquito transmission can attenuate *Plasmodium* virulence (Spence et al., 2013). Furthermore, the basic reproductive number (R_0) for malaria and other vector-borne diseases, which defines parasite fitness and transmission (Smith et al., 2012), predominantly includes parameters linked to vector-parasite interactions. Thus, understanding the ecology of vector-parasite interactions, and the extent to which parasite life history traits vary, is required to be able to explain the evolutionary potential of parasites, and the subsequent consequences for intervention success and infection dynamics (Oke et al., 2022) (see Chapter 2).

Variation in parasite traits influences virulence, transmission and the success of interventions such as antimalarial drugs (Mackinnon and Read, 1999a; De Roode et al., 2005; Schneider et al., 2012b; Goodman et al., 2016; Birget et al., 2018). For example, parasite genotypes with faster replication rates are more virulent to the vertebrate host, and have a higher competitive ability in genetically mixed infections (De Roode et al., 2005). Considering parasite traits in the context of life history theory can provide a framework for explaining why parasites do things the way they do, and how variation in traits can shape evolutionary processes (Reece et al., 2009; Neal and Schall, 2014a; Schneider and Reece, 2021). In particular, testing for phenotypic plasticity and quantifying genetic variation in parasite traits is required to infer how parasites might to evolve in response to environmental change, including control

measures such as drugs and vaccines, and vector control-induced changes to mosquito populations (Carter et al., 2013; Oke et al., 2022) (see Chapter 2). Furthermore, studying parasite responses to environmental variation under ecologically relevant settings across their whole life cycle is required to fully explain the evolutionary potential of parasite traits, and identify constraints that interventions could exploit.

Table 1.1. Examples of variation in malaria parasite traits across species (species), within species (genotype) and during the course of infection or when parasites encounter different types of host/vector (infection). Categories that have not been studied are highlighted in grey.

Trait	Examples of trait variation
Within-host	
Cell cycle duration	**Species:** Cell cycles are approximately 24 h for *P. chabaudi*, 48 h for *P. falciparum* and 72 h for *P. malariae* (Mideo et al., 2013) **Genotype:** A *P. chabaudi* genotype has a shorter cell cycle than other genotypes (Birget et al., 2019). **Infection:** *P. chabaudi* can speed up its cell cycle to maintain synchrony with host feeding rhythms (O'Donnell et al., 2021) and the *P. falciparum* cell cycle undergoes dormancy in response to drugs (Teuscher et al., 2010).
Synchrony	**Species:** *P. chabaudi* and *P. falciparum* are synchronous, but *P. berghei* and *P. yoelii* are asynchronous (Mideo et al., 2013). **Genotype:** More virulent *P. chabaudi* genotypes are less synchronous (Owolabi et al., 2024). **Infection:** *P. chabaudi* infections lose synchrony as parasite density increases (O'Donnell et al., 2021).
Burst size (merozoites per schizont)	**Species:** Burst size can range from 4 to 90 dependent on species (Schall, 1990; Kissinger et al., 2002; Reilly et al., 2007).

	Genotype: Burst size varies across *P. chabaudi* genotypes (Birget et al., 2019), and are higher in more virulent genotypes (Mideo et al., 2011). **Infection:** Burst size varies dependent on RBC age structure and density (Birget et al., 2019).
Red blood cell (RBC) preference	**Species:** *P. ovale, P. vivax* and *P. berghei* prefer to infect reticulocytes, *P. malariae* prefers to infect mature RBCs and *P. falciparum* and *P. chabaudi* can infect all RBC age classes (Cromer et al., 2006; Lim et al., 2016; Leong et al., 2021, 2022). **Genotype:** *P. chabaudi* genotypes and *P. falciparum* laboratory strains show differences in RBC preference (Antia et al., 2008; Mideo et al., 2011; Cai et al., 2020)**.** **Infection:** *P. falciparum* may become less selective as an infection progresses (Simpson et al., 1999), and *P. chabaudi* shifts preference from mature RBCs to reticulocytes during peak parasitaemia (Taylor-Robinson and Phillips, 1994).
Virulence	**Species:** Within the human malarias, *P. falciparum* causes the majority of deaths, followed by *P. vivax* and *P. knowlesi*, then *P. ovale* and *P. malariae* (Walker and Rogerson, 2023). **Genotype:** *P. chabaudi* genotypes vary in virulence to the vertebrate host (Mackinnon and Read, 1999a). **Infection:** *P. chabaudi* increases investment in asexual replication in response to co-infection (Pollitt et al., 2011b) and RBC availability is expected to limit virulence evolution (Pak et al., 2024).
Sex ratio	**Species:** *P. chabaudi, P. berghei* and *P. vinckei* exhibit different sex ratio patterns (Reece et al., 2009). **Genotype:** Sex ratio patterns differ across *P. chabaudi* genotypes (Reece et al., 2008). **Infection:** Sex ratio patterns vary over the course of an infection (Reece et al., 2009), and become less female-biased as the genetic diversity of infections increases in *P. chabaudi*, *P. mexicanum* and *P. falciparum* (Read et al.,

	1992; Reece et al., 2008; Neal and Schall, 2014b; Schneider et al., 2019).
Conversion rate	**Species:** Variation in conversion rate is observed across *P. chabaudi*, *P. vinckei* and *P. falciparum* (Carter et al., 2013). **Genotype:** *P. chabaudi* genotypes have different conversion patterns (Pollitt et al., 2011b), and differ in the extent of their conversion rate increase in response to anaemia (Birget et al., 2017). **Infection:** *P. chabaudi* and *P. falciparum* increase conversion in response to high drug doses (Buckling et al., 1997; Schneider et al., 2018a), *P. chabaudi* reduces conversion in response to low drug doses (Schneider et al., 2018a) and *P. chabaudi* increases conversion in response to anaemia (Birget et al., 2017).
Within-vector	
Gametogenesis (i.e. exflagellation)	**Species:** *P. gallinaceum, P. berghei, P. yoelii* and *P. falciparum* vary in their sensitivity to xanthureic acid, which stimulates gametogenesis (Arai et al., 2001)**.** **Genotype:** Unknown **Infection:** The number of male microgametes per gametocyte can be lowered by vigorous movement, and high temperatures inhibit exflagellation of male microgametes (Sinden and Croll, 1975).
Ookinete formation and development	**Species:** *P. vinckei* and *P. yoelii* are less efficient at forming ookinetes from gametocytes compared to *P. berghei* and *P. chabaudi* (Poudel et al., 2008). **Genotype:** Unknown **Infection:** Different numbers of *P. berghei* ookinetes are produced across different mosquito species (Vaughan et al., 1991), density-dependent effects regulate ookinete numbers (Sinden et al., 2007), and variation exists in ookinete gene expression within a midgut population (Witmer et al., 2021).
Ookinete invasion and oocyst prevalence	**Species:** *P. chabaudi* and *P. yoelii* have a more efficient ookinete to oocyst transition than *P. berghei* and *P. vinckei* (Poudel et al., 2008).

	Genotype: Differences in mosquito immune evasion across *P. falciparum* isolates (Molina-Cruz et al., 2012), and more virulent *P. chabaudi* genotypes transmit to mosquitoes at a higher rate (De Roode et al., 2005; Bell et al., 2006). **Infection:** Evidence for apoptosis of ookinetes to regulate infection density (Reece et al., 2011), and oocyst prevalence is reduced when *P. chabaudi* infects mosquitoes during night-time (Schneider et al., 2018b).
Oocyst density	**Species:** <10 oocysts in *P. falciparum*, <100 oocysts in *P. chabaudi* and >200 oocysts in *P. berghei* (Ferguson et al., 2003a; Spence et al., 2012; Pollitt et al., 2013; Bompard et al., 2020). **Genotype:** Limited evidence that more virulent *P. chabaudi* genotypes produce more oocysts (Ferguson et al., 2003a; Spence et al., 2012) and there are differences in densities across *P. falciparum* isolates (Molina-Cruz et al., 2015). **Infection:** Density can reduce over time due to mosquito immune function (Smith and Barillas-Mury, 2016) and there is density-dependent regulation of oocyst numbers (Sinden et al., 2007; Pollitt et al., 2013).
Oocyst growth rate	**Species:** Presumably different growth rates lead to different EIPs (see below) **Genotype:** Unknown **Infection:** Increased oocyst size when mosquitoes are provided with an additional blood meal (Shaw et al., 2020), oocysts become dormant in nutrient-limiting conditions (Habtewold et al., 2021) and oocyst size varies within a midgut (Andolina et al., 2024).
Sporogony duration (EIP)	**Species:** Under 7 days in *P. mexicanum* (Fialho and Schall, 1995), ~14 days in *P. chabaudi* (Spence et al., 2012) and *P. falciparum* (Shaw et al., 2022) and ~21 days in *P. berghei* (Bogale et al., 2021). **Genotype:** Limited evidence that EIP differs across *P. falciparum* isolates (Guissou et al., 2023). **Infection:** EIP speeds up when mosquitoes are provided with an additional blood meal (Shaw et al., 2020), sugar

	availability alters the EIP (Shiau et al., 2024) and a higher sporozoite burden correlates with a shorter EIP (Andolina et al., 2024).
Sporozoites per oocyst	**Species:** ~6500 for *P. yoelii*, ~10000 for *P. falciparum* (Kanatani et al., 2024) **Genotype:** Unknown **Infection:** Lipid deprivation reduces viable sporozoites per oocyst (Costa et al., 2018) and the number of sporozoites per oocyst can vary within a midgut (Andolina et al., 2024).
Oocyst rupture	**Species:** There are species-specific mechanisms of sporozoite escape from oocysts (Orfano et al., 2016). **Genotype:** Unknown **Infection:** Unknown
Sporozoite density	**Species:** Low sporozoite densities are more common in *P. yoelli* than *P. falciparum* (Kanatani et al., 2024). **Genotype:** Unknown **Infection:** Lipid deprivation reduces sporozoite quantity (Costa et al., 2018), sugar availability alters densities (Shiau et al., 2024), and infectivity to the vertebrate host is correlated with sporozoite density (Churcher et al., 2017; Aleshnick et al., 2020; Kanatani et al., 2024).
Sporozoite quality	**Species:** Sporozoite colonisation of the salivary glands differs between *P. yoelii* and *P. falciparum* (Kanatani et al., 2024), and *P. yoelii* sporozoites are 50-100 times more infective to mice than *P. berghei* (Briones et al., 1996). **Genotype:** Unknown **Infection:** Motility reduces as sporozoites age (Van Schuijlenburg et al., 2024) and lipid deprivation reduces sporozoite metabolic activity (Costa et al., 2018).
Sporozoite inoculum size	**Species:** ~800 *P. falciparum*, ~150 *P. yoelii* (Kanatani et al., 2024). **Genotype:** Unknown **Infection:** Unknown

1.5 Study system

Rodent malaria models are an ideal model system for applying evolutionary ecology to understand host-vector-parasite interactions and how parasite strategies contribute to the severity and spread of infections (Paul et al., 2003). This is because they are the best understood eukaryotic parasites, and are tractable systems for exploring the entire life cycle *in vivo*, where all parties (host, vector and parasite) can be experimentally manipulated. Furthermore, multiple genetically distinct clones of the rodent malaria *P. chabaudi* exist (Carter, 1978; Otto et al., 2014), allowing experimental designs to disentangle the contributions of genetic and environmental variation (e.g. common garden experiments). Understanding malaria parasite traits is also important because *Plasmodium* parasites and their relatives cause significant mortality and morbidity worldwide in humans, wildlife, and livestock (Garnham, 1966), and human *Plasmodium* parasites have been able to evolve resistance to all types of antimalarial drugs (Neafsey et al., 2008; Talman et al., 2019).

While rodent and human malaria parasite biology is fundamentally conserved, there are subtle differences between *Plasmodium* species (Simwela and Waters, 2022). However, life history theory can explain variation in life history strategies across diverse taxa. For example, evolutionary theory has been able to predict and explain sex ratio variation in a variety of organisms, including insects, birds and malaria parasites (West et al., 2002). Specifically, theory predicts that female-biased sex ratios are favoured when genetically related males complete for mates, because males can fertilise more than one female, so an equal sex ratio would result in wasteful excess male gametes (Nee et al., 2002). Indeed, several *Plasmodium* spp. can alter their sex ratio to become less female-biased as the genetic diversity of infections increases and relatedness reduces, so it would be very surprising if this is not the norm across the *Plasmodium* genus (Read et al., 1992; Reece et al., 2008; Neal and Schall, 2014b; Schneider et al., 2019). In addition, life history theory also predicts that the trade-off between survival and reproduction shapes the optimal investment into reproductive effort (Stearns, 1992). This has been experimentally demonstrated in free living organisms, including guppies (Reznick et al., 1990), and *P. chabaudi* (Birget et al., 2017; Schneider et al., 2018a). Thus, due to the generality of evolutionary theory, it is unlikely that human malaria parasites will be exceptions to these rules.

1.6 Explaining variation in within-vector traits

The premise of my thesis is that due to the extensive variation in environmental conditions experienced within the vector, it would be beneficial for parasites to adjust their development in response, to suit their circumstances. However, whether parasites can detect and actively respond to their within-vector circumstances, as evidenced in the within-host environment, has been overlooked. My experiments are based on observations that parasites can modulate their growth within the vector to maximise transmission. Firstly, ookinetes may be able to undergo apoptosis to regulate the density of mosquito infections, which is beneficial because parasites experience negative density-dependence (Arambage et al., 2009; Pollitt et al., 2010, 2013; Reece et al., 2011). Molecular studies have also identified variable candidate genes during the ookinete stage that may be able to respond to environmental cues (Witmer et al., 2021). Second, *P. falciparum* oocysts may undergo dormancy under stressful, nutrient-limiting conditions, restarting development if there is an influx of resources when their vector takes a blood meal (Habtewold et al., 2021). This could be adaptive if parasites can sense that their vector is unlikely to die imminently and will be able to take in more food (i.e. a blood meal), but maladaptive if vector lifespan is reduced and onward transmission is curtailed prior to additional food intake. In addition, in sub optimal conditions, a small subset of oocysts continue growing whilst the others halt development (Habtewold et al., 2021). This could maximise fitness if it allows limited resources to be allocated to a small proportion of oocysts and ensure that some sporozoites reach the salivary glands and allow onward transmission to occur. Third, oocysts respond to an influx of additional resources, by speeding up the EIP when mosquitoes are provided with an additional blood meal (Shaw et al., 2020; Kwon et al., 2021).

Disentangling to what extent these responses are due to resource constraints and/or adaptive parasite strategies is difficult. Exploring the extent to which parasite genotypes can shift their development in response to environmental changes, and the variation exhibited between genotypes is a useful starting point and has important consequences for parasite evolution (Oke et al., 2022). For example, if parasites can plastically adjust their EIP in response to cues for mosquito survival, this could maximise transmission potential in mosquito populations with reduced lifespan. However, parasites are likely to experience trade-offs between traits, so faster developing parasites may be of reduced quality and/or fewer numbers. Thus, key open questions remain regarding whether parasite genotypes exhibit variation in a variety of traits important for development and onward transmission, which is

addressed in Chapter 3, and the extent of plasticity for these traits in response to environmental change. Specifically, I consider how parasites modulate their growth in response to a change in nutritional resource availability in Chapter 4, and the trade-offs which may be occurring.

The need to understand the within-vector strategies of parasites is also becoming increasingly important because vectors are changing (Rivero et al., 2010; Couper et al., 2021; Oke et al., 2022) (see Chapter 2). Climate change and land use change are altering vector habitats and range distributions, which likely increases the within-vector environmental variation that parasites are exposed to. For example, *Aedes aegypti*, the vector of dengue and Zika virus, can adapt to tolerate higher temperatures (Dennington et al., 2024), facilitating survival across wider geographical ranges. In addition, vector control tool (VCT) use has altered the genotypes and phenotypes of mosquitoes, providing parasites with novel challenges to cope with and/or opportunities to exploit. For example, malaria parasites more frequently encounter vectors that can survive insecticide exposure, because VCTs are predominantly insecticide-based and mosquito populations have evolved resistance mechanisms in response (Ranson and Lissenden, 2016). There is evidence that parasites perform better in insecticide resistant mosquitoes, benefitting from increased oocyst density, growth and subsequently higher sporozoite density (Adams et al., 2023). However, there is a lack of consensus for how insecticide resistant mosquitoes affect parasite fitness, likely due to multiple resistance mechanisms at play which can affect parasites in different ways. In addition, *Anopheline* mosquitoes are shifting the time of day that they bite humans (see Chapter 5), and vector species composition is changing as a result of VCT use, because the major interventions target indoor, night-biting mosquito species (Moiroux et al., 2012; Sougoufara et al., 2016; Thomsen et al., 2017). Thus, in Chapter 2, I develop a framework to address the varying impacts of VCTs on parasites, by considering the overarching agents of selection that VCTs place on parasites, which can provide predictions for how parasites may evolve in response (Oke et al., 2022). Testing these hypotheses requires the extent of genetic variation, phenotypic plasticity and GxE in within-vector parasite traits to be assessed (Chapters 3 and 4). Additionally, the potential drivers of VCT-evasion strategies in mosquitoes and how they influence vectorial capacity (i.e. the entomological components of R_0) and parasite fitness requires investigation (Chapter 5). Finally, there is an urgent need for new tools to improve our ability to measure transmission potential and parasite fitness (Chapter 6).

1.7 Thesis outline and aims

In this thesis, I use a combination of lab and field experiments to investigate how variation in parasite life history traits within the vector, variation in vector traits, and interactions between the two, influence parasite transmission, fitness and evolutionary potential. My work has furthered our understanding of vector-parasite interactions from an evolutionary ecology perspective in the following ways:

- Providing an evolutionary ecology framework for investigating and predicting parasite evolution in response to vector control tool-induced changes in vectors (Chapter 2).
- Demonstrating that malaria parasite traits expressed in the vector exhibit strain-specific differences, suggesting that genetic variation exists for traits important for successful between-host transmission, which is the pre-requisite for evolution (Chapter 3).
- Revealing that phenotypic plasticity exists for both malaria parasite traits within the vector, and in vector foraging rhythms, in response to variation in mosquito nutritional resources. These chapters also highlight that how environmental variation (i.e. mosquito resource availability) can have contrasting effects on factors affecting between-host transmission (Chapters 4 and 5).
- Developing and testing a novel non-destructive assay to track malaria parasite development over time, to increase the tools available for investigating parasite fitness (Chapter 6).

Chapter 2. Vector control: agents of selection on malaria parasites?

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2.1 Abstract

Insect vectors are responsible for spreading many infectious diseases, yet interactions between pathogens/parasites and insect vectors remain poorly understood. Filling this knowledge gap matters because vectors are evolving in response to the deployment of vector control tools (VCTs). Yet, whilst the evolutionary responses of vectors to VCTs are being carefully monitored, the knock-on consequences for parasite evolution have been overlooked. By examining how mosquito responses to VCTs impact upon malaria parasite ecology, we derive a framework for predicting parasite responses. Understanding how VCTs affect the selection pressures imposed on parasites could help mitigate against parasite evolution that leads to unfavourable epidemiological outcomes. Furthermore, anticipating parasite evolution will inform monitoring strategies for VCT programs as well as uncovering novel VCT strategies.

Glossary 2.1.

Genotype-by-environment interaction (GxE): Different genotypes differ in the extent of their plastic response to a change in environmental conditions, demonstrating genetic variation for plasticity.

Gonotrophic cycle: The cycle of blood-feeding, egg development and oviposition of female mosquitoes.

Heritability: The proportion of phenotypic variance in a trait that has an additive genetic basis, which is a key determinant of the evolutionary potential of a trait.

Indoor residual spraying (IRS): An intervention that targets indoor-biting mosquitoes by coating indoor wall surfaces of a house with an insecticide that kills mosquitoes when they rest on these surfaces after feeding.

Insecticide-treated bednet (ITN): Bednets that have been treated with a pyrethroid insecticide and protect the user against biting mosquitoes. New generation bednets include additional chemistry, such as a pyrrole insecticide, the

synergist piperonyl butoxide (PBO) which enhances the efficacy of pyrethroids, or the insect growth regulator pyriproxyfen.

Life history trade-off: A trade-off exists when an increase in one life history trait is coupled to an unavoidable decrease in a second trait. Trade-offs can be mediated at the physiological level (e.g. competitive allocation of resources to different traits) and/or have a genetic basis (alleles having antagonistic pleiotropic effects or linkage disequilibrium between loci). Genetic trade-offs can constrain evolution.

Life history traits: Traits which affect organismal fitness. Life history theory is the branch of evolutionary theory developed to explain how selective forces shape the traits/strategies/phenotypes of multicellular organisms to optimise survival and reproduction. For multicellular organisms, the target of natural selection is usually considered as a single organism, but in single-celled parasites the target is best viewed as a single genotype within an infection, because the fitness interests of closely related parasite cells are aligned.

Phenotypic plasticity: The phenomenon by which a given genotype can produce different traits/phenotypes in response to a change in environmental conditions. Plasticity is considered adaptive when a plastic genotype has higher fitness following a (often predictable) change in environmental conditions compared to a non-plastic genotype, but plasticity can also be maladaptive or have a neutral impact on an organism's fitness.

Population: Group of individuals (i.e. parasite genotypes) that can interbreed.

Reaction norm: The shape of the relationship between the phenotypes (e.g. different values for a life history trait) produced by a specific genotype and the environmental conditions inducing these phenotypes. When the reaction norms for different genotypes are compared across the same environmental conditions, different slopes indicate genotype-by-environment interactions.

Sporogony: The obligate phase of sexual reproduction, development, and replication of *Plasmodium* parasites within the insect vector.

2.2 Why consider parasite evolution in response to vector control tools?

Vector control is one of the most effective methods to curb vector-borne diseases, with insecticide-based interventions predicted to have averted the majority of malaria cases since 2000 (Bhatt et al., 2015). However, because they reduce vector survival and reproduction, the continued and widespread use of chemical-based vector control tools (VCTs) has contributed to the evolution of insecticide resistance and evasion, particularly in *Anopheline* mosquito populations (Box 2.1) (Killeen et al., 2017; Huijben

and Paaijmans, 2018), and threatens progress towards malaria elimination targets (World Health Organisation, 2021). It is therefore not surprising that the responses of insect vectors to VCTs receives intensive investigation (Ranson and Lissenden, 2016; Carrasco et al., 2019). However, remarkably little attention has been paid to how VCTs alter parasite-vector-host interactions and how parasites are responding to the selection pressures imposed by the consequences of VCTs.

Just like drugs or vaccines, VCTs are an ecological perturbation that decreases parasite fitness by reducing vectorial capacity (the rate at which a vector can transmit a pathogen from a currently infectious case, Box 2.2) (MacDonald, 1956). History illustrates that attempts to reduce the survival and/or transmission of parasites/pathogens is readily met with counter-evolution. For example, malaria parasites have evolved resistance against all classes of antimalarial drugs (Hyde, 2005), and can alter **life history traits** (see Glossary 2.1) to partially compensate for fitness lost due to drug treatment (Schneider et al., 2018a). Parasites transmitted via vectors targeted by VCTs face diverse perturbations to their ecology [reviewed in (Rivero et al., 2010)]. In the short-term, at the start of a control program, parasites experience a dramatic drop in the health, abundance, and lifespan of vectors, and in the longer-term, parasites encounter vectors that have altered genotypes and phenotypes, as well as alternative vector species.

Predicting parasite responses to VCT-driven changes to their ecology requires: (i) uncovering how VCTs affect parasite fitness, both directly and indirectly via their impacts on vectors; (ii) establishing which aspects of VCTs impose constraints on parasite activities and/or provide opportunities to better exploit vectors; and (iii) considering how *de novo* mutation, standing genetic variation, and **phenotypic plasticity** can contribute to the adaptation of parasites to VCT-imposed alterations of parasite-host-vector-interactions. Here, we give an overview of (i) and (ii), which are more thoroughly covered in (Rivero et al., 2010), and we focus on providing a framework to address (iii), including monitoring and mitigation strategies.

Due to the wealth of knowledge about the effects of insecticide-based VCTs on *Anopheline* vectors of malaria, we focus on this system, but the concepts will be generalisable to other vector-borne infectious diseases and other VCTs, including tools currently in development, such as gene drives and endectocides (compounds administered to mammalian hosts to render blood meals toxic to mosquitoes) (Dabira et al., 2021; Namias et al., 2021). Moreover, opening the black box of parasite-vector

interactions may reveal how to make VCTs robust to clinically and epidemiologically unfavourable parasite counter-evolution as well as uncover new approaches for VCTs.

Box 2.1. Vector responses to insecticide-based VCTs

Mechanisms of resistance

Insecticide target site mutations reduce insecticide toxicity by causing structural modifications to target proteins, and include knockdown resistance mutations (*kdr*) in the *para sodium channel* gene (pyrethroid/DDT resistance), an *Rdl* gene mutation (dieldrin resistance) and an acetylcholinesterase enzyme (*ace-1*) mutation (organophosphate and carbamate resistance) (Alout et al., 2016). Increased metabolism and clearance via overexpression of detoxification gene families, including cytochrome P450-associated monooxygenases (P450s), glutathione S-transferases (GSTs) and carboxylesterases enhance insecticide detoxification (Ingham et al., 2021a). Other mechanisms include reducing insecticide penetration via a thicker cuticle (Balabanidou et al., 2016), and sequestration by chemosensory proteins in the legs (Ingham et al., 2020).

Insecticide exposure

Due to insecticide decay and insecticide-resistance (IR) mechanisms, exposure to sublethal insecticide doses occur. In the short-term, this stimulates changes to detoxification and redox metabolism gene expression (Ingham et al., 2021a, 2021b), and reduces host-seeking and blood-feeding (Glunt et al., 2018; Thiévent et al., 2019). Longer-term effects include reduced survival of both IR and insecticide-susceptible (IS) mosquitoes (Viana et al., 2016; Glunt et al., 2018), but lifespan is not affected in some highly resistant populations (Hughes et al., 2020). Furthermore, older mosquitoes are more susceptible to insecticides (Jones et al., 2012).

Methods for evasion

Avoidance is an alternative to coping with insecticides and occurs by blood foraging less frequently or at times of day when hosts are not protected by bed nets (Carrasco et al., 2019), in the early evening (Thomsen et al., 2017) or early morning (Moiroux et al., 2012). ITNs target anthropophilic species that bite indoors at night, rather than less specialist feeders that bite at any time of day and/or outdoors. Thus, sustained ITN use is associated with increased outdoor biting (Russell et al., 2011)

and resting (Kreppel et al., 2020), as well as seeking higher proportion of blood meals from non-human vertebrate hosts (Ndenga et al., 2016; Kreppel et al., 2020).

Further considerations

Identifying the genetic basis and **heritability** of IR traits is challenging for complex behaviours such as a biting time-of-day, habitat choice, and host preference, but some behavioural resistance strategies are heritable (Main et al., 2016; Govella et al., 2023). In general, mechanisms conferring resistance are costly when expressed in the absence of insecticides. For example, biochemical resistance is associated with costs across both larval and adult stages (Otali et al., 2014; Assogba et al., 2016; Nkahe et al., 2020; Tchouakui et al., 2020), including reduced fecundity and lifespan, differing across vector genotypes and resistance mechanisms (Okoye et al., 2007; Alout et al., 2016). Trade-offs may limit avoidance; changes to biting time-of-day affects reproductive schedule (O'Donnell et al., 2019) and may cause 'jet lag' between feeding rhythms and other circadian regulated processes, such as detoxification and immune responses (Rund et al., 2016), increasing susceptibility to insecticides at certain times of day (Balmert et al., 2014). Furthermore, blood meals from non-preferred host species reduces mosquito fitness (Lyimo et al., 2013), due to differences in haematological properties (Emami et al., 2017).

2.3 VCTs: Mosquito responses and the consequences for parasites

Insecticide-based VCTs, such as **insecticide-treated bednets (ITNs)** and **indoor residual spraying (IRS),** are highly effective but their widespread deployment and limited chemical nature has selected for the evolution of multiple insecticide resistance (IR) mechanisms (Toé et al., 2014). Resistance mechanisms can be biochemical, morphological, or behavioural (Carrasco et al., 2019), and underpinned by genetic evolution and/or deployment of pre-existing adaptive phenotypic plasticity (APP) (summarised in Box 2.1).

During the mosquito phase of the *Plasmodium* life cycle (**sporogony,** Figure 2.1A), *Anopheline* vectors undergo multiple **gonotrophic cycles**, temporally coupling activities that must be undertaken by parasite and vector (Werling et al., 2019) (Figure 2.1B). Thus, parasites will likely be directly exposed to insecticides at regular intervals during sporogony when host-seeking mosquitoes interact with ITNs/IRS, as well as facing the effects that insecticides have on vectors (including alterations to lifespan, resource acquisition and allocation, metabolism, oxidative state, and immune

responses, Box 2.1). Furthermore, the consequences of insecticide exposure include disrupted blood-feeding schedules and changes in the species composition of vectors, which also impact host-parasite interactions during onwards transmission.

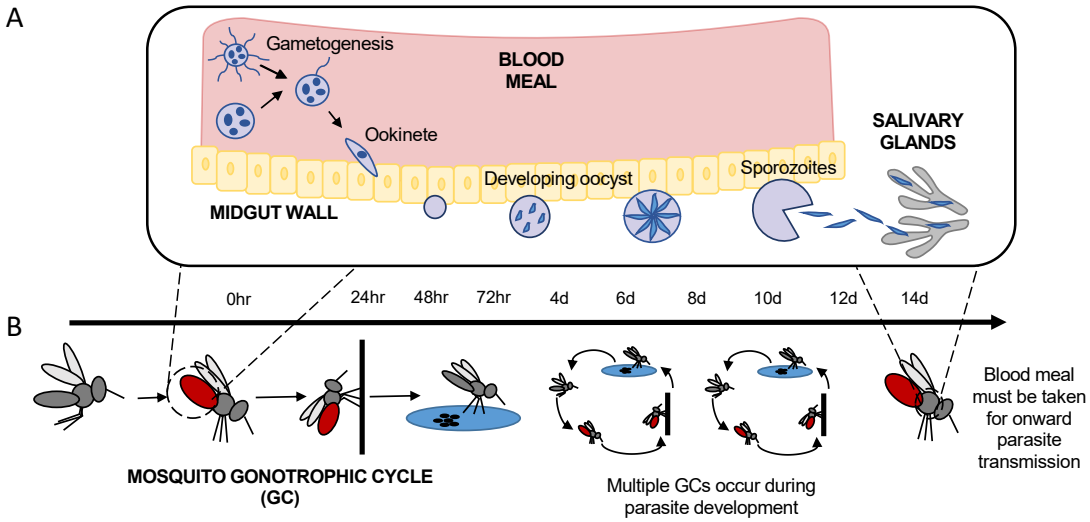

Figure 2.1. Mosquito and malaria parasite ecology are closely interlinked.

A) Malaria transmission is initiated in the vertebrate host when a small proportion of parasites during each intraerythrocyctic developmental cycle commit to producing sexual stages (gametocytes). Upon ingestion in a blood meal, parasites undergo sexual reproduction, where gametocytes rapidly differentiate into gametes, undergo a single round of sexual reproduction, and develop into motile ookinetes. Ookinetes traverse the midgut wall and become oocysts, within which parasites undergo many rounds of asexual replication over multiple days to produce thousands of sporozoites. Sporozoites are released upon oocyst egress and must migrate through the haemocoel to the salivary glands where they reside until injected into a new host upon blood feeding. B) Female *Anopheles* mosquitoes seek a blood meal from a human. After blood-feeding, mosquitoes spend approximately 48-72 hours resting while their eggs develop and then seek an oviposition site to lay eggs, sometimes ingesting a sugar meal for energy before they begin host seeking again. The gonotrophic cycle (blood-feed, egg development, oviposition) can repeat approximately 2-3 times during the period of oocyst development. VCTs can affect the gonotrophic cycle in a variety of ways and therefore affect parasite as well as mosquito ecology.

The additive, antagonistic, or interacting effects of VCT-driven ecological perturbations on parasite-vector-host interactions are poorly understood, with many key open questions (Rivero et al., 2010). The few studies available suggest the individual impacts of these factors on transmission are complex and that variation in IR mechanisms and vector genotype add further complexities (Okoye et al., 2007; Alout et al., 2016). In this section, we outline how interactions with vectors that possess IR mechanisms, exhibit altered behaviours, and belong to different species, all affect parasite ecology and relate these impacts to transmission and vector competence (Box 2.2).

Box 2.2. R_0, vectorial capacity and transmission dynamics

R_0, also known as the Ross-MacDonald equation, describes the expected number of infected hosts generated from a single infected host in a completely susceptible population. It is defined using the equation (Smith et al., 2012):

$$R_0 = \frac{ma^2bc}{(-\ln p)r}p^v$$

The equation for vectorial capacity derives from the above. It excludes *r*, the daily rate that each human recovers from infection because it contains the purely entomological concepts of R_0 (Smith et al., 2012):

$$V = \frac{ma^2bc}{(-\ln\ p)}p^v$$

Density of vectors per vertebrate hosts (m). Transmission is positively correlated to the number of vectors per host and is shaped by the reproductive output of vector species and how this interacts with environmental factors.

Human-biting rate (a). The rate that humans are bitten varies across vector species due to species specificity in host preference and whether preference depends on the availability of different kinds of host (Takken and Verhulst, 2013).

Vector competence (bc). The proportion of bites by an infectious mosquito that infect a human (*b*) and the probability that a mosquito becomes infected after biting an infected human (*c*) makes up vector competence (Cator et al., 2020). The ability of a parasite to survive within the vector during development into the form which is

infective to a new human host reflects the combined effects of parasite infectivity to vectors and vector susceptibility (Lefèvre et al., 2013).

Duration of parasite extrinsic incubation period (EIP) (v). The time it takes for the parasite to develop from the point of ingestion to the form infective to a new human host is shaped by parasite intrinsic factors (Ohm et al., 2018) as well as environmental temperature (Shapiro et al., 2017) and resource availability within the vector (Shaw et al., 2022). Because *v* and *p* interact exponentially, small changes in EIP can dramatically affect vectorial capacity.

Daily probability of adult survival (p). The EIP lasts for a long proportion of vector lifespan and transmission requires that the vector survives throughout this period.

All vectorial capacity parameters (even including *m*) are a product of how parasites and vectors interact and subject to alteration by VCTs. Based on observations in the literature, Table 2.I below illustrates potential impacts of the consequences of VCTs on these parameters. Increases are denoted by "+", reductions by "-", and scenarios that are yet to be investigated or for where specific details matter and general principles are unlikely are indicated by "?". Particularly noteworthy is that the effects of VCTs on parasite contributions to vectorial capacity are largely unknown.

Table 2.I. The consequences of VCT use and their potential effect on vectorial capacity parameters.

Impact of VCT on vectors	**Potential change to vectorial capacity parameter**			
	+	**-**	**?**	**No change**
Reduction in population size	-	*m, a*	-	*bc, v, p*
Insecticide exposure	-	*m, a, bc, p*	*v*	-
Insecticide resistance mechanisms	*m, p*	*p*	*a, bc, v*	-
Altered resource allocation	-	-	*a, bc, v, p*	*m*
Change in species composition	-	*m, a, bc*	*v, p*	-
Biting time shifts	*a*	*m, p*	*bc, v*	-
Change to outdoor biting	*a*	*m, p*	*bc, v*	-

2.3.1 Insecticide exposure and IR mechanisms

Due to the near ubiquitous nature of insecticide resistance, modern parasites frequently encounter vectors altered by IR mechanisms (Box 2.1). These parasites

also face the effects of direct exposure to insecticides through vectors that have been exposed to sublethal doses; this occurs because IR vectors can withstand contact with high doses and insecticide-susceptible (IS) vectors may survive contact with degraded insecticide. Because the duration of sporogony (the extrinsic incubation period, EIP) is long relative to mosquito lifespan, even small effects of IR vectors and/or insecticide exposure on the survival and development of parasites can have large effects on parasite fitness (Box 2.2).

Few studies have considered the direct consequences of insecticide exposure for parasite fitness but observations include reduced mating and/or impaired early development (Hill, 2002; Alout et al., 2014a; Kristan et al., 2016). Pyrethroid insecticides, which are neurotoxic, are efficacious against some Apicomplexans (Symington et al., 1999), but the direct impact on *Plasmodium* is currently unknown. The multiplicity of insecticide resistance mechanisms could provide protection for the parasite from the direct action of the insecticide through lowering concentration of the toxic insecticide; however, indirect effects of these mechanisms on the parasite are likely to be costly.

Insecticide exposure is also expected to reduce transmission by inhibiting host-seeking activity of IR mosquitoes, lengthening the gonotrophic cycle, and potentially decreasing mosquito lifespan (Thiévent et al., 2019). However, whilst some studies demonstrate that IR mechanisms reduce the intensity of malaria infections (Lo and Coetzee, 2013), others show that IR mechanisms make mosquitoes more susceptible to infection (Alout et al., 2013; Ndiath et al., 2014), or have no effect (Wolie et al., 2021). The lack of consensus for how IR mosquitoes affect parasite fitness (Minetti et al., 2020) is likely due to the wide range of IR mechanisms (Box 2.1) that can also interact in complex ways, the difficulties of controlling for the confounding effects of insecticide exposure and age in field studies, and because malaria infection may exacerbate mosquito susceptibility to insecticides (Alout et al., 2014b).

2.3.2 Altered resource allocation

Like all organisms, mosquitoes have a finite amount of resources, and face **life history trade-offs** where they must differentially allocate resources between life history traits (e.g. immune defences versus reproduction, antioxidants versus immune defences). Life history trade-offs may explain in part the reduced fecundity of IR mosquitoes compared to their susceptible counterparts (Tchouakui et al., 2020), as well as their differences in microbiota (Cansado-Utrilla et al., 2021), immune gene

expression (Vontas et al., 2005), respiration rate (Ingham et al., 2021b) and lifespan (Brown et al., 2020). Additionally, most IR mechanisms involve over-production of proteins, which requires the investment of resources. For example, to ameliorate the oxidative costs of overexpressed P450s (Box 2.1), mosquitoes may invest relatively more in redox management, which could trade off against investment in immune defences, fecundity and/or lifespan.

How mosquitoes divide resources between IR mechanisms and managing their consequences versus other traits is likely to have complex consequences for parasites. For example, oxidative stress through the production of reactive oxygen species (ROS) negatively affects parasites (Kumar et al., 2003) and so IR involving ROS could provide a form of immune defence against infection. However, if these mosquitoes reduce their fecundity to cope with infection and/or insecticide exposure (Tchouakui et al., 2020), resources may be released for parasites to scavenge, enhancing parasite productivity. Furthermore, if investment in fecundity is prioritised over non-ROS forms of immune defence, parasites also benefit. Yet, if maintaining physiological health and fecundity come at the expense of lifespan, parasites may suffer from premature vector mortality. Thus, the net effects of mosquito life history strategies on parasite transmission may pivot on mechanisms that affect reactive oxygen species (ROS) levels in potentially contrasting ways (Ingham et al., 2021a, 2021b). This complexity is highlighted by the contrasting effects of the detoxification enzymes, the P450s and GSTs (see Box 2.1), which elevate and reduce oxidative stress respectively (Rivero et al., 2010).

2.3.3 Behavioural avoidance

Insecticide evasion behaviours of mosquitoes include biting at a time-of-day humans are not protected by ITNs, resting outdoors, and blood feeding from alternative host species (Box 2.1). In one sense, behavioural avoidance of ITNs and IRS is beneficial for parasites, because by reducing the chance of insecticide-induced vector mortality, parasites have more opportunities for onwards transmission. However, this benefit may be eroded if behavioural avoidance delays blood-feeding because parasites may receive resources from the blood meal less often or at less useful points during sporogony (Costa et al., 2018; Shaw et al., 2020). Similarly, if mosquitoes shift to feeding on non-human hosts, they may receive a blood meal they are less capable of utilising and so, both vectors and parasites become resource limited (Lyimo et al., 2013).

Daily rhythms dominate malaria transmission; parasites enter and exit the vector at specific times-of-day. Parasites are confronted with daily rhythms in mosquito physiology, including immune responses and insecticide detoxification (Rund et al., 2011) for the duration of infections. If day-biting leads to mosquito rhythms being temporally decoupled from the parasite's developmental schedule, parasites could benefit if key transitions in the life cycle, such as ookinete migration shifts to a time of day when mosquito immune responses are suppressed (Rund et al., 2016). However, temporal dysregulation of the mosquitoes' own rhythms as a consequence of a shift in the timing of blood feeding could reduce mosquito fitness and consequently, decrease vector population size and reduce lifespan (O'Donnell et al., 2019). Environmental rhythms may also play a role; when reared under realistic daily temperature regimes, mosquitoes feeding in the early evening are more competent vectors (Suh et al., 2020). This is likely due to an interaction between the effects of mosquito rhythms on susceptibility and the temperature-sensitive mating of parasites.

How blood feeding rhythms affect transmission is further complicated by the role of daily rhythms exhibited by hosts and parasites. For example, *An. stephensi* mosquitoes infected in their rest phase (i.e. day time for nocturnal mosquitoes) are more susceptible to *P. chabaudi*, but this seeming advantage is negated by gametocytes being less infective during the day-time (Schneider et al., 2018b). Whilst host rhythms do not affect transmission to mosquitoes in lab models (O'Donnell et al., 2019), this might not be the case in nature. For example, dengue replicates faster and exhibits a shorter EIP when its *Aedes aegypti* vector receives a blood meal with a high blood glucose concentration (Weng et al., 2021). In keeping with this, malaria parasite gametogenesis and mating are glucose-hungry processes (Talman et al., 2014) and day-feeding *Anophelines* will coincide with the day-time peak in blood glucose of human hosts. However, any advantage of entering a mosquito at an unusual time of day might be eroded by entering a new human host at this time-of-day because mammalian hosts are generally more susceptible to infection in their rest phase (night for humans) (Westwood et al., 2019). However, predicting whether transmission is affected by VCT-induced shifts in the timing of blood-seeking requires information on how much vector biting rhythms can shift.

2.3.4 Shifts in vector species

A consequence of ITNs and IRS primarily targeting indoor, night-biting, anthropophilic species is that transmission shifts to less anthropophilic vector species which are experiencing less of a decline in areas of high ITN use (Tedrow et al., 2019; Sanou et

al., 2021). This imposes a change in both the composition and the relative abundance of different species that can be used as vectors. The constraints and opportunities presented to parasites by alternative vectors are even less well studied than the previously discussed consequences of VCTs. However, because vector species differ in their activity rhythms and their preferences for host species, it is likely that parasites may encounter similar problems as described in the previous section (Carnevale and Manguin, 2021; Sangbakembi-ngounou et al., 2022).

Other important behaviours, such as the number of blood meals taken during sporogony, also vary between species (Guelbéogo et al., 2018), affecting both transmission opportunities and the number of parasite genotypes that sequentially coinfect mosquitoes (Pollitt et al., 2015). Furthermore, sporogony is less productive when vectors take blood meals from novel host species (Emami et al., 2017). In addition to inter-specific variation in behaviours that affect transmission, differences in immune regulation (Simões et al., 2017) across vector species affect vector competence. While *Plasmodium* has adapted to evade the immune system of its local vector across its wide geographical range, parasites perform less well in non-sympatric vectors (Molina-Cruz et al., 2015). Thus, if parasites are decreasingly able to rely on a single vector species, vector specialists will not transmit as successfully as generalists. Large scale comparative experiments are required to reveal which parasite-vector interactions underpin successful sporogony across vector species and to predict the impacts of vector shifts on transmission.

2.4 Parasite responses to the consequences of VCTs

Assuming evolution and/or APP allows parasites to alter transmission traits in response to the consequences of VCTs, how might parasites be changing? In an ideal world, parasites would infect long-lived, frequently blood-feeding vectors, and produce large numbers of highly infective sporozoites after a short EIP. However, constraints and trade-offs limit parasite transmission, many of which are imposed by their hosts and vectors. The latter include host/vector immune responses, the type and amount of host/vector resources available, vector lifespan, and interactions with other organisms sharing the vector, all of which are likely to be altered by VCTs as illustrated in the previous sections (also see Box 2.2).

Parasite traits underpinning transmission include the density and sex ratio of gametocytes, which are traits expressed within the host, and all the stages of sporogony which include mating and zygote development, ookinete migration, oocyst

development, sporozoite egress and migration to the salivary glands (Figure 2.1A). In the following sections, we illustrate how these traits could be altered in response to the consequences of VCTs and identify the likely costs, constraints, and limits to these responses. Each parasite trait can be fit into the framework outlined in Box 2.3 and Figure 2.I., in which the environments refer to aspects of vector traits before and during deployment of a VCT. Rather than exploring responses to each of the impacts of insecticide VCTs discussed above, we collate them into key over-arching agents of selection on parasites in the following sections.

Box 2.3. Parasite evolution framework

How, and over what time scales, parasite populations can respond to VCTs are unknown. Yet, this information can direct monitoring programmes to mitigate against the most likely, or concerning, parasite responses. Just as vector responses to VCTs occur via evolution by natural selection and APP, parasite responses can also take these forms.

If an ecological perturbation imposes a strong enough selective pressure and there is genetic variation for fitness-related traits within the **population**, evolution occurs. Genetic variation is well documented for transmission traits that parasites express in the host (e.g. investment into, and sex ratio of, gametocytes (Schneider and Reece, 2021)), though heritability is infrequently reported. Observations are consistent with intraspecific genetic variation for within-vector parasite phenotypes (e.g. infectivity to a given mosquito strain (Molina-Cruz et al., 2015) and effects on mosquito survival and fecundity (Ferguson et al., 2003a)) in both lab models and natural infections, and interspecific genetic variation in EIP (Lefevre et al., 2017).
APP in traits expressed during sporogony has been overlooked, but EIP is sensitive to resource availability (Hien et al., 2016; Shapiro et al., 2016; Werling et al., 2019; Shaw et al., 2020), temperature (Shapiro et al., 2017) and parasite density (Childs and Prosper, 2020). Whether this is due to the parasite adopting APP or simply due to physiological constraints imposed by the vector upon parasite development is unknown. Given the utility of APP within-host (Schneider and Reece, 2021), it would be surprising if parasites were unable to use APP to cope with the variation in vectors they encounter.

If parasites use APP to alter traits during sporogony to maintain fitness according to, for example, whether their vector is affected or not by a VCT, predicting evolution becomes more complex. This is because APP itself is subject to evolution by natural selection, and APP can facilitate or constrain evolution in non-mutually exclusive ways, dependent on the **reaction norms** exhibited by genotypes in a population, and whether there are **genotype-by-environment interactions** (Figure 2.I) (Chevin et al., 2010). Ecological perturbations generally increase the extent of genetic variation exposed to selection, elevating evolutionary potential (Paaby and Rockman, 2014). In contrast, APP could constrain evolution; by mitigating against the loss of fitness caused by VCTs, APP weakens selection on parasites (Chevin et al., 2010). Selection can also be constrained if trade-offs between traits have a genetic basis and/or if multiple traits face opposing selection pressures (Stearns, 1989).

Testing for APP and quantifying genetic variation remain key objectives to infer the consequences of VCTs for parasite evolution. Highly controlled laboratory studies can inform which traits and genetic markers should be prioritised for monitoring in the field, as well as provide forecasts to integrate into epidemiological models.

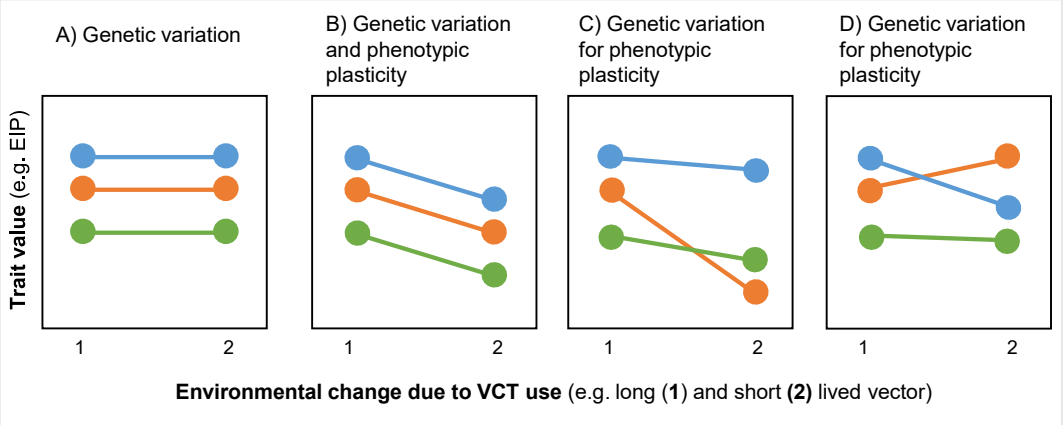

Figure 2.I. The evolutionary potential of parasites. A hypothetical parasite population is composed of three different genotypes, which adopt different values for a fitness-related trait in different environmental conditions. The spread of intercepts indicates the extent of genetic variation exposed to selection in each environment and the slope dictates the degree of plasticity of each genotype (i.e. how much a genotype's trait changes across environments). The four possible patterns are: genotypes exhibit no plasticity but do differ from each other (A), all

genotypes are plastic and respond by the same extent (B) and by differing extents (C), and genotypes respond in different manners (D). Applying this to a scenario where the trait is the EIP and in condition 1 (long-lived vector unaffected by a VCT) a long EIP is optimal, but in condition 2 (vector with reduced lifespan due to impacts of a VCT) a shorter EIP returns higher fitness, we can predict that: (i) The relative abundance of the genotypes changes because the fitness ranks alter (blue is fittest and green is least fit in long-lived vectors, but in short-lived vectors, green generally performs best); (ii) Compared to scenarios (A) and (D), selection is more effective in (C) because the spread of the genotypes' trait values is greater in condition 2 than 1; (iii) Aside from the switch in fitness ranks, selection is constrained in (B) because plasticity allows all genotypes to alter EIP in the direction that maintains fitness.

2.4.1 Infection genetic diversity

Reducing transmission is expected to lower the probability of genetically mixed infections and reduce the number of co-infecting genotypes in vectors and hosts (Karl et al., 2016). The multiplicity of infection (MOI) determines the degree of competition each genotype faces, and within-host competition is costly enough to select for APP in reproductive strategies and favour more virulent parasites (Bell et al., 2006; Schneider and Reece, 2021). Thus, theory predicts that a reduction in MOI allows parasites to increase investment in transmission (Schneider and Reece, 2021) and may lead to less severe infections. Furthermore, because within-host competition exacerbates the fitness costs of drug resistance, a reduction in MOI lessens this constraint. This suggests that parasites and drug resistance alleles may spread better, but infections would be less severe.

Whether and how MOI affects within-vector ecology and shapes parasite evolution are open questions. Intuition suggests that competition between unrelated parasites genotypes is more intense within the vector due to limited amounts of nutrients, space, and time, compared to within a human. If so, a reduction in MOI will also release parasites from within-vector competition, but the opposite has been noted in certain circumstances (Pollitt et al., 2015). Heterospecific interactions, such as between parasites and the mosquito microbiome, can also reduce parasite transmission via altered mosquito immune defences (i.e. "apparent competition") (Gao et al., 2020). Thus, IR-induced changes to the microbiome (Omoke et al., 2021) may drive selection for immune evasion or strategies to perturb microbiota composition.

2.4.2 Vector longevity

Whilst insecticide exposure generally reduces lifespan, parasites may encounter mosquitoes with a broader distribution of lifespans because IR mechanisms affect longevity differentially (Brown et al., 2020). To cope with increased variation in vector lifespan, it would be beneficial for parasites to use APP to alter their EIP to match vector lifespan on a mosquito-by-mosquito basis. If parasites can alter the EIP, this is most likely achieved by curtailing the oocyst stage, which is relatively long compared to sexual reproduction and sporozoite migration (Figure 2.1). However, if parasites are unable to detect reliable cues for vector lifespan then bet-hedging is the best strategy (Starrfelt and Kokko, 2012), which could be achieved by producing oocysts that burst at different times to generate a range of EIPs. Furthermore, moderation of EIP via APP according to the circumstances of individual mosquitoes would allow parasites to avoid the costs of a shorter EIP when they encounter mosquitoes unaffected by VCTs.

How much the EIP can shift is unclear; since EIP is close to mosquito life expectancy, it is assumed that constraints imposed by the vector and/or benefits to the parasite must be maintaining this duration (Koella, 1999). Perhaps a short EIP means fewer rounds of mitosis are undertaken within oocysts, reducing sporozoite numbers (Gerald et al., 2011). Alternatively, speeding up replication to maintain sporozoite production during a short EIP might interfere with the rate of lipid acquisition, resulting in less infective sporozoites (Costa et al., 2018). However, these costs might be worth paying in dire circumstances; ensuring some level of transmission from a short-lived vector is better than none.

An alternative view is that resource availability mediates the EIP. For example, when additional resources are available, oocysts can afford to speed up development (Shaw et al., 2020) but must avoid exploiting mosquito resources too rapidly and risk premature vector mortality, as observed for dengue (Ye et al., 2016). Whereas, in resource-limited conditions, oocysts exhibit dormancy, with growth rescued by providing additional resources (Habtewold et al., 2021). In this scenario, dormancy is an adaptive strategy when vectors are resource-limited but likely to survive long enough to gain additional resources, but maladaptive in short-lived vectors with little chance of survival.

2.4.3 Resource allocation and immune defences

Due to VCT use leading to evolution of IR mechanisms, exposure to insecticides, and changes to vector behaviours and species composition, parasites are likely to encounter mosquitoes with differing resource allocation patterns (Shaw et al., 2022). For example, pyriproxyfen is an insect growth regulator added to new generation ITNs which directly impacts mosquito reproduction. Allocation to reproduction has complex effects on parasites; lower investment in reproduction associates with a lower oocyst burden but faster development (Werling et al., 2019) suggesting that parasites scavenge resources during/after egg development (Costa et al., 2018). Thus, changes in mosquito resource allocation (due to trade-offs) or acquisition (due to altered gonotrophic cycles) could favour parasites that adjust EIP and/or that can manipulate mosquito reproductive processes. Interactions between different IR mechanisms and resource allocation to various forms of immunity are likely to result in multiple, potentially conflicting, selection pressures on parasites. Such complexity might constrain the evolution of mean trait values, and instead, favour APP in which parasites adjust vector exploitation activities (e.g. rate of oocyst growth) according to the type and strength of immune responses mounted by individual mosquitoes. For example, a stronger immune response may select for faster replicating (more harmful) parasites as observed in lab models (Shaw et al., 2022), as well as increased immune evasion. Such a strategy would be costly in low-resourced mosquitoes with a suppressed immune response that will die prematurely if exploited too aggressively. In which case, undergoing a form of programmed cell death to regulate the intensity of infection, which has been observed for ookinetes, is beneficial (Reece et al., 2011).

As well as facing IR-driven changes to the physiology of preferred vector species, parasites encounter different immune responses between alternative vector species. For example, mosquito immune responses exert their greatest impact on ookinetes traversing the midgut, and haplotypes of the surface protein *Pfs47* allow *P. falciparum* isolates to avoid immune detection by their local vector (Molina-Cruz et al., 2015). Whilst it is unlikely that a given parasite genotype will be able to effectively evade immune responses across multiple mosquito species, parasite genotypes with the "wrong" haplotype for a particular mosquito species can infect other species, albeit at a lower prevalence and intensity (Molina-Cruz et al., 2015). Thus, selection to adapt to an alternative vector or become generalists might drive the evolution of *Pfs47* haplotypes that are not optimal to infect any individual vector population but do reduce detection across multiple species.

2.4.4 Mosquito behaviours

As well as IR mechanisms and insecticide exposure, shifts to less anthropophilic vector species and altered biting time expose parasites to mosquitoes with different behaviours. A consequence of transmission via less anthropophilic mosquito species is that parasites will encounter non-human hosts more often. Alongside adaptation to novel vector species, parasites may become host-generalists or undertake host-shifts because the receptors that parasites use for red blood cell invasion vary across vertebrate species (Lim et al., 2013). An alternative solution is to manipulate vectors to preferentially bite humans and manipulate humans to attract preferred vector species (Robinson et al., 2018; Vantaux et al., 2021). Vector manipulation abilities would also benefit parasites facing vectors with longer gonotrophic cycles (Osoro et al., 2022) to ensure transmission opportunities as soon as sporozoites become infectious.

Altered biting time of day (via behavioural avoidance or vector species shifts) cause parasite activities to be out-of-synch with rhythms within the vector and with the abiotic environment. First, parasites may have to adjust their developmental schedule to align with mosquito rhythms. For example, if ookinete invasion of the midgut is most successful during the night-time (i.e. approx. 20 hours post blood meal), ookinetes may have to accelerate development or wait until the following evening to invade. Both options are likely to incur costs; fast ookinetes are likely to be lower quality and waiting may increase the risk of being digested. Second, day-biting forces parasites to undergo gametogenesis and fertilisation during warmer parts of the circadian cycle than night-biting. High thermal sensitivity during early sporogony is widely observed across human, murine and avian malaria parasites (Ball and Chao, 1964; Vanderberg and Yoeli, 1966; Noden et al., 1995). Thus, if day-biting mosquitoes do not find cool places to rest following blood feeding, high temperatures may be a physiological constraint that parasites cannot adapt to cope with. Therefore, VCTs that are most likely to be evaded by vectors biting in the daytime have good potential to be robust against parasite counter-evolution.

In areas of seasonal transmission, a consequence of VCTs reducing vector density is that fewer mosquitoes are likely to be present at the start of the transmission season to stimulate parasites into increasing transmission investment, as observed for avian malaria parasites (Cornet et al., 2014). If a similar phenomenon occurs in human parasites, they may not receive stimulation by sufficient mosquitoes until further into the transmission season, leading to a shorter transmission window. In this case,

parasites would benefit by becoming more sensitive to mosquito bites or evolving to use a different proxy for seasonality, such as seasonal changes in host hormones, to schedule their transmission activities according to vector availability. However, whether parasites detect the activities of vectors or use alternate proxies for seasonality is unknown. Alternatively, this problem may strengthen selection for manipulation of humans to be more attractive to mosquitoes.

2.4.5 Coping with insecticide exposure

Whether direct exposure to insecticide agents imposes selection pressure on parasites is unclear, but does raise the question of whether parasites can evolve resistance to insecticides, as they do against antimalarial drugs. Ookinetes appear to be sensitive to insecticides (Kristan, 2018) and may contact insecticides picked up the previous night during blood feeding. On one hand, because this stage is short-lived, protecting it from insecticide toxicity might be possible (for example, by dormancy), especially because exposure appears to be relatively transient (Hill, 2002), depending on how much this increases the risk of being digested. On the other hand, ookinete survival is a major bottle neck in sporogony suggesting there are many ecological constraints ookinetes are unable to overcome. Understanding the risks of direct exposure to insecticides faced by parasites requires knowledge of how long clearance takes in IR mosquitoes. This is further complicated by the addition of chemicals, such as piperonyl-butoxide (PBO) and chlorfenapyr, to new generation ITNs which prolong the window of insecticide efficacy on mosquitoes and potentially on parasites.

For parasites to encounter an insecticide, their vector must have encountered it too, and it is possible that coping with both direct exposure plus the effects of insecticides on the vector constrain parasite evolution. Furthermore, analogous to antimalarial drug resistance and IR in mosquitoes, parasite IR will involve fitness costs. Thus, unless the IR mechanism is expressed only during sporogony or occurs via APP, absence of insecticide pressure in a vertebrate host will cause IR mutants to be outcompeted by sensitive parasites. If parasites do evolve to evade insecticides, the options available are likely to depend on the stage of sporogony when detrimental exposure occurs. The most obvious tactic would be to enter dormancy until the insecticide is no longer active. This is unlikely to be feasible during sexual reproduction and may be too costly for ookinetes, but could be deployed following exposure during the oocyst stage.

2.5 Addressing parasite evolution in response to VCTs

Predicting and monitoring parasite responses to VCTs should not be a secondary aim of programmes, only to be undertaken once the primary goal of vector control is achieved. Clearly, this is a huge and multidisciplinary challenge, but progress is possible. Laboratory studies are an efficient way to test for proof-of-concept for fundamental evolutionary predictions as well as determine how the most promising hypotheses should be tested in endemic settings, given the parameters and nuances of natural infections. Animal models are invaluable for estimating parasite fitness and testing whether there are clinical consequences of genetic correlations underpinning traits expressed in sporogony and traits expressed in the vertebrate host. Genetic variation and APP (Box 2.3) are hard to assess without undertaking common-garden experiments that have considerable statistical power, which is possible using lab and semi-field settings. For example, comparing traits across parasite genotypes when each is represented by multiple replicate infections in which the age/strain/condition of vectors, density/sex ratio of gametocytes, and environmental perturbations are tightly controlled. Selection experiments where parasites are serially 'passaged' through different types of vector, can also reveal how parasite genotypes and phenotypes evolve and provide genetic markers for field monitoring. However, the most acute need is to ascertain the role that individual parasite traits play in vector competence and transmission (Box 2.2).

2.6 Concluding remarks

VCTs reduce vectorial capacity and therefore transmission. Keeping transmission under control requires anticipating and preventing parasite counter-evolution, especially given that almost all parameters underpinning vectorial capacity are indirectly or directly influenced by parasite activities (Box 2.2) (Lefevre et al., 2017). Thus, how VCTs affect selection on, and the potential evolutionary responses of, parasites is a major knowledge gap. We have explored the diverse manners in which VCTs affect malaria parasite transmission and illustrated how parasites could meet these challenges. The scenarios we cover are not an exhaustive treatment but highlight the diverse potential outcomes of VCTs for parasite ecology and evolution.

How parasites have evolved to exploit vectors and cope with the constraints they impose have largely remained a black box since mosquito transmission was discovered over a century ago. Yet, a better understanding of vector-parasite interactions in the presence and absence of VCTs will reveal approaches that are most robust to parasite counter-evolution, including directing interventions to target

the most evolutionarily constrained parasite traits, as well as informing programmes designed to monitor parasite responses to VCTs.

2.7 Outstanding questions

- How do the myriad ways that vector biology is affected by VCTs directly and/or indirectly affect parasite interactions with insect vectors and human hosts? *E.g. how does insecticide resistance affect resource allocation in vectors, and how does this affect parasite development?*
- Do the ecological perturbations caused by VCTs impose constraints on parasite activities that reduce fitness and/or can VCTs provide opportunities for parasites to exploit to enhance fitness? *E.g. do the costs of insecticide resistance cause vectors to mount weaker immune responses against parasites, facilitating transmission?*
- How much heritable genetic variation is there for parasite traits that are exposed to selection as a result of VCTs, and are these traits subject to genetic and resource allocation trade-offs? *E.g. do parasite genotypes that have the fastest development in the vector produce the fewest host-infective stages?*
- Will parasite plasticity and genotype-by-environment interactions facilitate or constrain parasite evolutionary responses to selection driven by VCTs? *E.g. if parasites possess the ability to plastically adjust development time according to variation in vector lifespan, does this reduce the strength selection to alter developmental duration?*
- How do parasite traits expressed in the vector link to clinical/epidemiological outcomes for human hosts? *E.g. are parasite genotypes with a faster development in the vector less infective/virulent to human hosts?*
- Can knowledge of how plasticity and evolution help parasites cope with the consequences of VCTs be harnessed to improve the efficacy of VCTs? *E.g. if VCTs with differing modes of action impose antagonistic selection pressures on parasites, could combining these tools in specific combinations retard unfavourable parasite evolution?*

Chapter 3. Strain-specific differences in between-host transmission of the malaria parasite, *Plasmodium chabaudi*

3.1 Abstract

Understanding how natural selection shapes pathogen populations is key for anticipating and preventing clinically unfavourable counter-evolution in response to interventions. Genetic variation underlying phenotypic traits which shape a pathogen's fitness is required for natural selection to occur. For example, *Plasmodium* parasites, which are the causative agents of malaria, display genetic variation for traits that underpin within-host survival and infectivity to mosquito vectors. However, the extent of variation in within-vector (sporogonic) traits is poorly understood, despite multiple developmental life stages occurring in the vector and completion of sporogony being required for onward transmission from vector to host. Using three genetically distinct clones of the rodent malaria *P. chabaudi*, which exhibit genetic variation in within-host traits, we investigate whether there are genotypic differences in traits expressed within the vector and in onward transmission from mosquito to vertebrate host. We find that the genotypes vary in the duration of time needed to complete sporogony, the number and productivity of within-vector developmental stages, and patterns of onward transmission to new hosts. We suggest that these differences may be due to genotypes resolving life history trade-offs in different ways to optimise between-host transmission, and hypothesise how this variation could shape parasite evolution. Given the myriad changes in mosquito populations resulting from vector control tools, the anticipated effects of climate change on transmission, and the drive to develop transmission blocking interventions, it is increasingly important to assess the evolutionary potential of vector-borne pathogens and parasites to adapt to changes in the behaviours, phenotypes and genotypes of vectors.

3.2 Introduction

Pathogen evolution poses an ongoing risk to human, animal and plant health. Therefore, understanding how natural selection shapes the traits of pathogen populations is essential for predicting the success of interventions to treat infections and prevent their spread. Life history theory has provided insight into how natural selection shapes the phenotypes (i.e. traits, behaviours, strategies) expressed by organisms to optimise their survival and reproduction, as well as explaining why

considerable variation in phenotypes is maintained within populations (Roff, 1992; Stearns, 1992). Life history theory is usually applied to multi-cellular organisms but can also explain phenotypes of parasites (including pathogens), often providing unexpected insight into their strategies for within-host survival and between-host transmission. For example, *Isospora* parasites exit their bird host at a specific time-of-day to avoid desiccation and damaging UV radiation (Martinaud et al., 2009), bacteriophages adjust their lysis time in response to competition with other phage strains within a host bacterial cell (Leggett et al., 2013), long-term relationships between hosts and parasitic worms has led to the evolution of immune evasion strategies (Viney and Cable, 2011), and co-infection of the host with different malaria parasite genotypes selects for the evolution of higher virulence (De Roode et al., 2005). Life history theory informs how individual organisms should allocate finite resources between phenotypic traits that trade off against each other, such as the allocation of resources to growth/maintenance occurring at the expense of investment into reproduction (Stearns, 1989; Froy et al., 2017). For macroparasites such as nematode worms (Viney and Cable, 2011), it is clear what constitutes an 'individual'. However, parasites that replicate within a host are analogous to a colony of social insects, where clonally related parasites within an infection are the 'individual', representing the comparable target for natural selection (West et al., 2006; West and Kiers, 2009).

Like any organism, parasites adapt genetically to environmental change via advantageous alleles, either in the form of new mutations, or pre-existing (standing) genetic variation within a population (Barrett and Schluter, 2008), with natural selection favouring the variants producing the phenotypes with the highest fitness for the current, or most often encountered, environment (Lefevre et al., 2017). For parasites, their 'environment' includes the conditions they encounter within their host and vector (in the case of vector-borne parasites). Fitness-determining aspects of the within-host/vector environments include immune responses, access to nutritional resources, the presence of co-infecting parasites, and interventions such as drugs, vaccines and insecticides (Reece et al., 2009; Huijben and Paaijmans, 2018). *Plasmodium* parasites, which are the causative agents of malaria, demonstrate that investigating parasite life history traits within the vertebrate host can reveal genotype-specific phenotypes and sophisticated strategies that maximise fitness (i.e. survival and transmission) under differing environmental conditions (Reece et al., 2009; Schneider and Reece, 2021). Many of these phenotypes have a genetic basis and are therefore a target for natural selection; for example, *Plasmodium* genotypes –

studied in rodent malaria models, cultured human parasites and wild lizard hosts – vary in traits that underpin asexual replication rate. Genetic variation in within-host survival traits is medically relevant because asexual replication rate dictates how virulent *Plasmodium* is to the vertebrate host (Mackinnon and Read, 1999a; Eisen and Schall, 2000; Reilly et al., 2007; Birget et al., 2019), more virulent genotypes have a competitive advantage (De Roode et al., 2005) and are less sensitive to antimalarial drugs (Schneider et al., 2012b). Within-host sexual reproduction traits, which influence transmission success, such as the proportion of asexual parasites committing to form sexual stages (gametocytes) and the gametocyte sex ratio (proportion of gametocytes that are male), also display genetic variation (Graves et al., 1984; Reece et al., 2008; Pollitt et al., 2011b; Schneider et al., 2018a). This variation may be maintained by natural selection; for example, different sex ratios may reflect disparities across genotypes in the number of gametes produced by male gametocytes, determining which sex ratio maximises mating success (Gardner et al., 2003; Reece et al., 2008). However, whether malaria parasites exhibit genetically based differences in traits expressed in their mosquito vector, and whether this variation can be adaptive (i.e. fitness maximising), are unknown.

Within their vector, malaria parasites undergo sporogony, which involves several sequential developmental stages over a period of approximately 10-20 days (Ohm et al., 2018; Howick et al., 2019; Childs and Prosper, 2020). Sporogony begins with gametogenesis and fertilisation to produce ookinetes in the midgut of the vector, ookinetes traverse the midgut wall and form oocysts, where extensive replication occurs to form infectious sporozoites, and ends when sporozoites egress from oocysts and migrate to the salivary glands. Onward transmission from the vector to a new host cannot occur without the successful completion of these processes, which requires exploitation of vector resources, evasion of immune responses, and for the mosquito to live long enough. The length of time it takes for within-vector development to be completed is termed the extrinsic incubation period (EIP). *Plasmodium* exhibits significant interspecific (between-species) variation in within-vector sporogonic traits; for example, the EIP is much shorter in the lizard malaria *P. mexicanum* than in other species (less than 7 days versus 10 – 20 days), potentially due to its sandfly vector having a shorter lifespan than a mosquito (Fialho and Schall, 1995). Furthermore, oocyst and sporozoite densities differ across species, likely in part due to differences in immune evasion and their ability to utilise vector resources (Kwon et al., 2021). For example, *P. falciparum* and *P. chabaudi* usually produce <10 oocysts and <100 oocysts per midgut, respectively (Ferguson et al., 2003a; Spence et al., 2012;

Bompard et al., 2020), compared to *P. berghei* which can reach oocyst densities between 200-500 (Pollitt et al., 2013). The number of sporozoites inoculated when a mosquito bites a host varies in a manner that correlates positively with host size, with *Anopheles stephensi* inoculating 5-fold more sporozoites into the skin when infected with the human parasite *P. falciparum* compared to when infected with the rodent parasite *P. yoelii* (Kanatani et al., 2024).

Despite the completion of sporogony being vital for life cycle completion, whether intraspecific (within-species) variation exists in sporogonic traits expressed by parasites has received little attention (Lefevre et al., 2017), with the majority of studies focussing on infectivity during transmission from host to vector (Lambrechts et al., 2005; Molina-Cruz et al., 2012). For example, genotypes of the rodent malaria *P. chabaudi* which are more virulent to the host achieve a higher prevalence (i.e. the proportion of mosquitoes that become infected) and oocyst burden (i.e. the number of oocysts per mosquito midgut) when transmitting to *Anopheles stephensi* (Ferguson et al., 2003a). Parasite genotypes can also differentially affect mosquito life history traits important for vectorial capacity, including mosquito lifespan and fecundity (Ferguson et al., 2003a). These parasite genotype-specific effects can also vary based on mosquito condition or co-infection (Ferguson and Read, 2002; Ferguson et al., 2003b), demonstrating environmental dependence of parasite traits. Such "phenotypic plasticity", in which parasites adjust their phenotypes in response to different environmental conditions, can also be genotype-specific. Adaptive plasticity in within-host traits is well documented; for example, *P. chabaudi* adjusts investment into gametocytes and their sex ratio according to the genetic diversity of infections and resource availability, with genotypes adjusting these traits in manners that depend on their competitive ability (Reece et al., 2008; Pollitt et al., 2011b; Schneider et al., 2018a). Given the degree of genetic variation and adaptive plasticity in *Plasmodium* phenotypes expressed in the host, it would be surprising if analogous variation is not observed within the vector. While this has been overlooked, there is suggestive evidence that EIP varies across *P. falciparum* isolates (Guissou et al., 2023). Assessing the evolutionary potential of parasites to adapt to variation in the kinds of vector they encounter requires knowledge about the extent of variation between genotypes for sporogonic traits (i.e. genetic variation), how plastic these traits are in response to different within-vector conditions (i.e. environment effects), and whether genotypes exhibit differing levels of plasticity (i.e. genotypes-by-environment interactions).

The evolutionary potential of parasites matters because mosquito vectors are changing. For example, the evolutionary responses of mosquitoes to insecticide-based control tools include alterations to resource allocation, immune responses, mosquito lifespan and biting time-of-day (Rivero et al., 2010; Oke et al., 2022), as well as shifts in the species composition of *Anopheline* vector populations. All of these factors are likely to be selective drivers of sporogonic traits exhibited by parasites. For example, insecticide exposure reduces mosquito lifespan in insecticide-resistant mosquitoes (Viana et al., 2016), which may select for parasites that can speed up sporogony to ensure onward transmission. To assess the potential for genetic variation in malaria parasite traits expressed throughout sporogony and during onward transmission from vector to host, we studied three genetically distinct clones of *P. chabaudi* (The European Malaria Reagent Repository, University of Edinburgh, UK). We focussed on sporogonic traits because they are more likely to be under parasite genetic control than indirect effects on mosquito traits (i.e. mosquito fecundity or lifespan) that affect vectorial capacity. We reveal that key parasite transmission traits differ across genotypes, providing preliminary support for genetic variation across a variety of sporogonic traits and in infectivity after onward transmission from vector to host. We discuss the implications of genotype differences for parasite fitness, and hypothesise that genotypes may resolve resource allocation trade-offs in different ways to maximise their transmission. Finally, we consider how genetic variation in sporogonic traits could influence parasite evolution in response to changes in vectors driven by vector control tools.

3.3 Methods

We conducted a series of experiments to track parasite development throughout sporogony and during onwards transmission from vector to host for three clones of the rodent malaria parasite *P. chabaudi* (The European Malaria Reagent Repository, University of Edinburgh, UK), which are genetically distinct according to electrophoretic enzyme analysis (Carter, 1978) and exhibit high genotypic diversity (Otto et al., 2014). We chose genotypes AJ, AS, and ER because they vary in fitness-related traits exhibited in the vertebrate host phase of the life cycle. Specifically, genotype ER replicates to a higher peak parasitaemia in the blood, 1.25-fold higher than AJ and 2.9-fold higher than AS. This results in ER being more virulent to hosts as measured by weight loss and anaemia, followed by AJ and then AS (Mackinnon and Read, 1999a; Ferguson et al., 2003a; Mackinnon et al., 2005). Furthermore, each asexual replication cycle of ER is approximately 1 hour faster than for AJ and AS

(Birget et al., 2019). These genotypes also vary in reproductive strategies, exhibiting differing patterns in the proportion of asexual parasites committing to producing sexual stage gametocytes (conversion rate), likely due to differences in resource acquisition between genotypes (Pollitt et al., 2011b). In particular, conversion rate is approximately 4-fold lower in genotype AJ compared to ER and AS (Pollitt et al., 2011b; Carter et al., 2013). Genotype ER produces approximately 7.5- and 16-fold more gametocytes than AS and AJ throughout infections (Mackinnon and Read, 1999a), and they also alter gametocyte investment and sex ratio throughout infections in different ways, illustrating genotype-by-environment interactions (Reece et al., 2008; Schneider et al., 2018a).

3.3.1 Vectors, hosts and parasites

While the natural vector for *P. chabaudi* is unknown (Stephens et al., 2012), *An. stephensi* is an excellent model vector because it is able to transmit a range of avian, rodent and human *Plasmodium* parasites (Ferguson and Read, 2002; Alavi et al., 2003; Kanatani et al., 2024). *Anopheles stephensi* SD500 mosquitoes were housed under standard insectary conditions of 26°C, 70% relative humidity and a 12L:12D cycle (Spence et al., 2012). Upon emergence, we provided adult mosquitoes with *ad libitum* access to 8% fructose solution. Four days before transmission from mice to mosquitoes, we treated mosquitoes with antibiotics (0.05% gentamicin) for 48 hours, and fructose solution was supplemented with 0.05% para-aminobenzoic acid (PABA) to aid parasite development (Jacobs, 1964). We transferred adult female mosquitoes to experimental pots (n=15 pots, n=90 mosquitoes per pot), and starved all mosquitoes for 24 hours prior to transmission from mice to mosquitoes.

Vertebrate hosts (for both transmission to, and from, mosquitoes) were 8-10 week old C57/Bl6J mice housed under standard 12L:12D conditions with *ad libitum* access to a standard RM3 pelleted diet (801700, SDS, UK) and drinking water supplemented with 0.05% PABA. We treated all mice (n=15) used to transmit parasites to mosquitoes with 125mg/kg phenylhydrazine (PHZ) via intraperitoneal (IP) injection prior to infection to enhance gametocyte production. Three days after PHZ treatment, we infected each mouse with $1x10^7$ *P. chabaudi* ring stage parasitised red blood cells by IP injection. Mice were infected with one of three genotypes; ER (n=5), AS (n=5) or AJ (n=5). We took blood smears and red blood cell (RBC) counts to test for variation across a variety of within-host parasite metrics on the day of transmission. We collected data from Giemsa-stained blood smears on the proportion of RBCs which

were (i) reticulocytes, (ii) infected with asexual parasites and (iii) infected with gametocytes. We calculated reticulocyte, asexual and gametocyte densities by multiplying these proportions by the total RBC density per µl blood. Transmission to mosquitoes occurred on day four post-infection, with one mouse (infected with AJ) excluded because it did not harbour gametocytes (n=5 (ER), n=5 (AS), n=4 (AJ), total n=14).

To transmit parasites from host to vector, we anaesthetised infected mice (50mg/kg ketamine hydrochloride and 0.5mg/kg medetomidine in PBS) and placed each mouse on a single pot of mosquitoes, allowing mosquitoes to blood feed for 20 minutes. Mosquitoes were 5-10 days old when receiving their infected blood meal and were given 8% fructose supplemented with 0.05% PABA daily thereafter. On day 1 post-infectious blood meal (pIBM), we removed unfed mosquitoes, and on day 3 pIBM, we provided an oviposition site for egg laying (a small petri dish lined with filter paper and filled with water) within each pot.

3.3.2 Traits expressed during sporogony

To test whether the genotypes exhibited different trait values during sporogony, we quantified the: (i) prevalence, (ii) burden, (iii) size, and (iv) variation in size (coefficient of variation), of oocysts within a midgut, and (v) infectivity of gametocytes to mosquitoes (oocyst burden divided by gametocyte density), all on day 7pIBM, and the (vi) prevalence and (vii) burden of sporozoites in the salivary glands on days 8, 9, 11 and 14 pIBM (Figure 3.1A).

For oocyst metrics, we anaesthetised n=8 mosquitoes per pot on ice, dissected their midguts and stained them in 0.25% mercurochrome. We photographed midguts under x40 magnification, to quantify prevalence, burden and size of oocysts using ImageJ (Schneider et al., 2012a). For sporozoite prevalence and burden, we anesthetised a subset of mosquitoes on ice (n=10 per pot) at each time point and bisected them following Foley *et al* (Foley et al., 2012). We stored head/thorax samples at -20°C until DNA extraction and sporozoite quantification by quantitative PCR (qPCR). We extracted DNA using a modified version of the CTAB protocol from Chen *et al* (Chen et al., 2010) as described in Schneider *et al* (Schneider et al., 2018b), eluted DNA in 30ul water and used a qPCR targeting the gene *PSOP1* (PCHAS_0620900) (Wargo et al., 2006) to quantify sporozoites. Quantification of parasite genomes was determined by comparing the threshold cycle (Ct) against a standard curve for each

P. chabaudi genotype, generated from DNA extracted from microscopy-quantified blood stage parasites from each of the three genotypes.

3.3.3 Completion of the malaria life cycle

To investigate onward transmission from vector to host, we transferred a subset of infected mosquitoes (n=12) from each pot into new pots on day 14 pIBM, and an anaesthetised naïve mouse (50mg/kg ketamine hydrochloride and 0.5mg/kg medetomidine in PBS) was placed on each new pot (n=5 (ER), n=5 (AS), n=4 (AJ), total n=14). After allowing mosquitoes to feed for 25 minutes, we removed mice and administered a reversal agent (10% Antisedan in PBS) via sub-cutaneous injection. One mouse bitten by ER-infected mosquitoes did not respond to the reversal agent and was culled. Following transmission feeds by infected mosquitoes, we sampled mice (n=4 (ER), n=5 (AS), n=4 (AJ), total n=13) 4, 6 and 8 days later, taking blood smears and RBC counts to follow asexual density dynamics (Figure 3.1B). We began at day 4 to ensure detectable parasitaemia, because the life cycle involves a 2-day replication phase in the liver before parasite egress into the blood stream (Stephens et al., 2012). To test for differences in sporozoite loads between mosquitoes in different pots and infected with different genotypes, we collected mosquito head/thorax samples from those that blood-fed, and extracted DNA and quantified parasites by qPCR as described above.

3.3.4 Statistical analysis

We used R v. 4.1.3 to perform data analysis. For transmission from host to vector, we analysed within-host data (asexual densities, gametocyte densities, RBCs and reticulocyte densities) using linear models with genotype as a main effect. To test for the impact of genotype on both oocyst prevalence and the proportion of naïve mice that became infected during onward transmission, we used binomial generalised linear models (glm). We used linear mixed models to test how genotype impacted (i) oocyst burden, (ii) oocyst size variation within a gut, (iii) the infectivity of gametocytes to mosquitoes, (iv) the correlation between oocyst size and oocyst burden, and (v) oocyst diameter in infected mosquitoes. We fitted a binomial generalised linear mixed model (glmm) to test the impact of genotype on sporozoite prevalence across day pIBM. Zero-inflated negative binomial glmms (*glmmTMB*, (Brooks et al., 2017)) were used to investigate how genotype impacted (i) sporozoite burden across day pIBM (with a zero-inflation parameter applied to day pIBM), (ii) the correlation between

oocyst burden and sporozoite burden on day 14 pIBM, and (iii) the sporozoite burdens of mosquitoes used for onward transmission to naïve mice. To account for non-independent measures within mosquito pots and mosquito guts respectively, we added a random effect of pot or a nested random effect of mosquito identity within mosquito pot. We used a negative binomial glm to investigate the impact of genotype on the total sporozoite load that naïve mice were exposed to during onward transmission. We defined total sporozoite load as the sum of the residual sporozoite burdens of mosquitoes that fed on each naïve mouse, because residual sporozoite burden per mosquito is positively correlated to the number of expelled sporozoites and infection probability (Churcher et al., 2017; Andolina et al., 2024; Kanatani et al., 2024). Finally, we used a lmm with a $\log_{10}$ transformation to investigate whether genotype impacted within-host parasite dynamics after onward transmission, with mouse ID as a random effect.

We minimised all models using likelihood ratio tests, and AICc for non-nested models, and tested for conformation to assumptions using *DHARMa* (Hartig, 2022), or *easystats* (Makowski et al., 2020) for models with nested random effects. Oocyst burden and infectivity data were square-root transformed to meet assumptions of normality and homogeneity of variance. We present predicted means and SEMs from minimised models (predict() function, *lme4*, (Bates et al., 2015)). For lmms/glmms generated using *lme4*, SEMs were estimated using parametric bootstrapping (bootMer function), and we used model predictions to fit linear or logistic regression curves. We ran post hoc pairwise comparisons using the Tukey method using the *emmeans* package (Lenth, 2023) whenever the minimal model contained a significant effect.

3.3.5 Ethics statement

All animal procedures complied with the UK Home Office regulations (Animals Scientific Procedures Act 1986; SI 2012/3039; PPL PP8390310) and were approved by the University of Edinburgh.

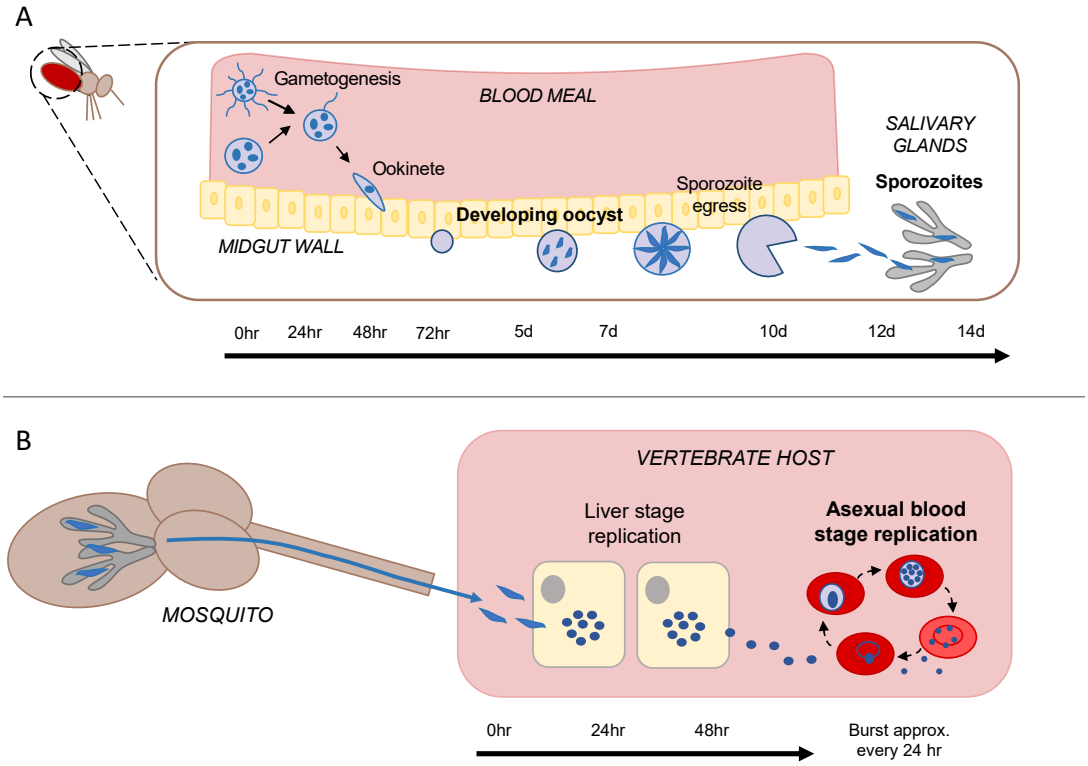

Figure 3.1. Parasite sporogonic traits, with approximate timings post-mosquito bite, expressed during transmission from host to vector (A) and from vector to host (B). We quantified the densities and prevalences of stages highlighted in bold. To test for variation in the duration of time taken for sporozoite migration to the salivary glands, we sampled this stage over multiple days (from day 8 to day 14).

3.4 Results

3.4.1 Within-host traits

On the day of transmission to mosquitoes, RBC density did not differ across genotypes ($F_{2,11}$=0.65, p=0.54) (Figure 3.2A). However, reticulocyte densities did differ ($F_{2,11}$=6.43, p=0.014), with AS infected hosts exhibiting approximately 1.6 and 1.3 fold more reticulocytes than AJ and ER, respectively, which did not differ from each other (Figure 3.2B, Table 3.1). We also found that AS produced 2.2-fold fewer asexual stages ($F_{2,11}$=13.1, p=0.0012) compared to AJ and ER, which had similar densities (Figure 3.2C, Table 3.1). In contrast, while genotypes AJ and AS exhibited similar gametocyte densities, ER produced 3-fold more ($F_{2,11}$=17.9, p<0.001) (Figure 3.2D, Table 3.1).

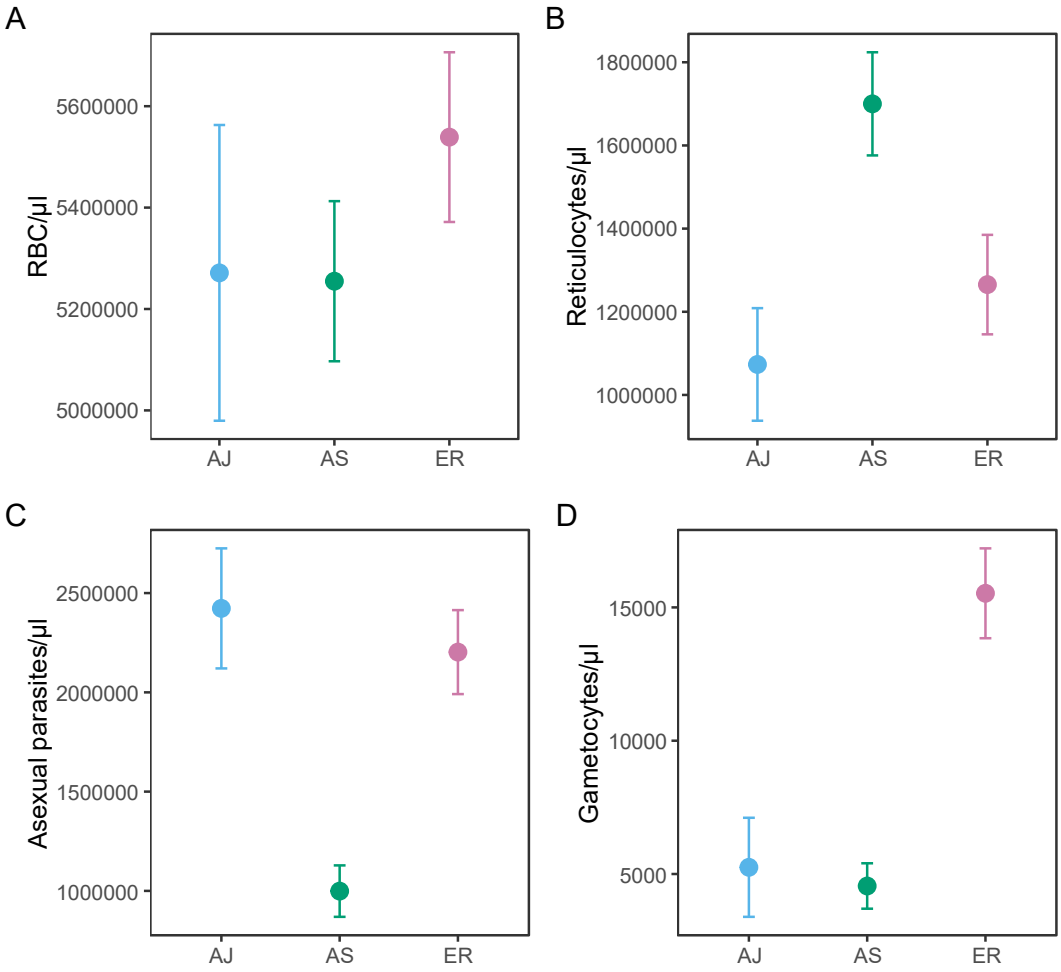

Figure 3.2. Within-host metrics for three *P. chabaudi* genotypes on the day of transmission to mosquitoes. Data presented are mean ± SEM for total red blood cell density (A), reticulocyte density (B) asexual parasite density (C) and gametocyte density (D).

3.4.2 Sporogony differs across genotypes

Despite differences in gametocyte density, all genotypes infected mosquitoes at a similar rate, with an overall oocyst prevalence of approximately 95% (χ^2_2=1.26, p=0.53) (Figure 3.3A). However, oocyst burden differed between genotypes; mosquitoes infected with genotype ER had approximately 8-fold more oocysts (sqrt burden: 14.0±1.0) (χ^2_2=25.2, p<0.001) than AS and AJ (sqrt burden: AS 4.2±1.0, AJ 5.8±1.1), which did not differ from each other (Figure 3.3B, Table 3.1). Per-gametocyte infectivity to mosquitoes was similar across genotypes with a marginal trend for ER's gametocytes to be more infective (0.11±0.01) than AS's (0.07±0.01) (χ^2_2=5.40, p=0.07) (Figure 3.3C). We also found that genotype ER's oocysts were the

largest (χ^2_2=10.4, p=0.006); approximately 13% larger (35.5±0.8μm) than AS oocysts (31.3±0.9μm) and 9% larger than AJ oocysts (32.6±1.0μm), which did not differ from each other (Figure 3.3D, Table 3.1). Even a small difference in oocyst diameter may be biologically significant because by being spherical, changes in oocyst diameter result in large changes in volume. Despite differences in oocyst burden and average size, we found oocysts within a gut varied equally in size across the genotypes (albeit a marginal trend for less variation within AS-infected guts, χ^2_2=5.96, p=0.051) (Figure 3.3E), and there was no correlation (i.e. potential trade-off) between the number and size of oocysts across any of the genotypes (χ^2_2=0.15, p=0.77) or overall (χ^2_1=2.52, p=0.11) (Figure 3.3F).

To investigate whether the EIP differed across genotypes, we compared patterns of sporozoite accumulation within salivary glands. Our monitoring window (day 8 –14 pIBM) captured when approximately 25 to 95% of mosquitoes harboured sporozoites, revealing that by day 11 pIBM, more ER-infected mosquitoes had accumulated sporozoites than AS-infected mosquitoes (reaching 89±4% for ER compared to 68±7% for AS) (χ^2_2=8.21, p=0.017), but not AJ-infected mosquitoes (79±6%) (Figure 3.4A, Table 3.1). The number of sporozoites in salivary glands followed similar patterns to prevalence; starting similarly low across the genotypes but ER reached 5-fold higher burdens by day 11 pIBM ($3.1x10^5$±$9.2x10^4$) compared to AS and AJ (AS: $6.4x10^4$±2.1 $x10^4$, AJ: $5.3x10^4$±1.7 $x10^4$) (χ^2_6=18.1, p=0.0061), which did not differ from each other (Figure 3.4B, Table 3.1).

Finally, we found a positive correlation between oocyst and sporozoite burden (χ^2_1=19.0, p<0.001) that followed the same pattern across genotypes (χ^2_2=1.07, p=0.58) (Figure 3.5, Table 3.1). Differences in sporozoites per oocyst across genotypes were not significant (χ^2_2=1.99, p=0.37). While each additional oocyst led to more sporozoites, this pattern was non-linear with diminishing returns; lower oocyst burdens (~40; as observed for AS/AJ) yield approximately 2700 sporozoites per oocyst, whereas higher oocyst burdens (~230; as observed for ER) yield approximately 1900 sporozoites per oocyst.

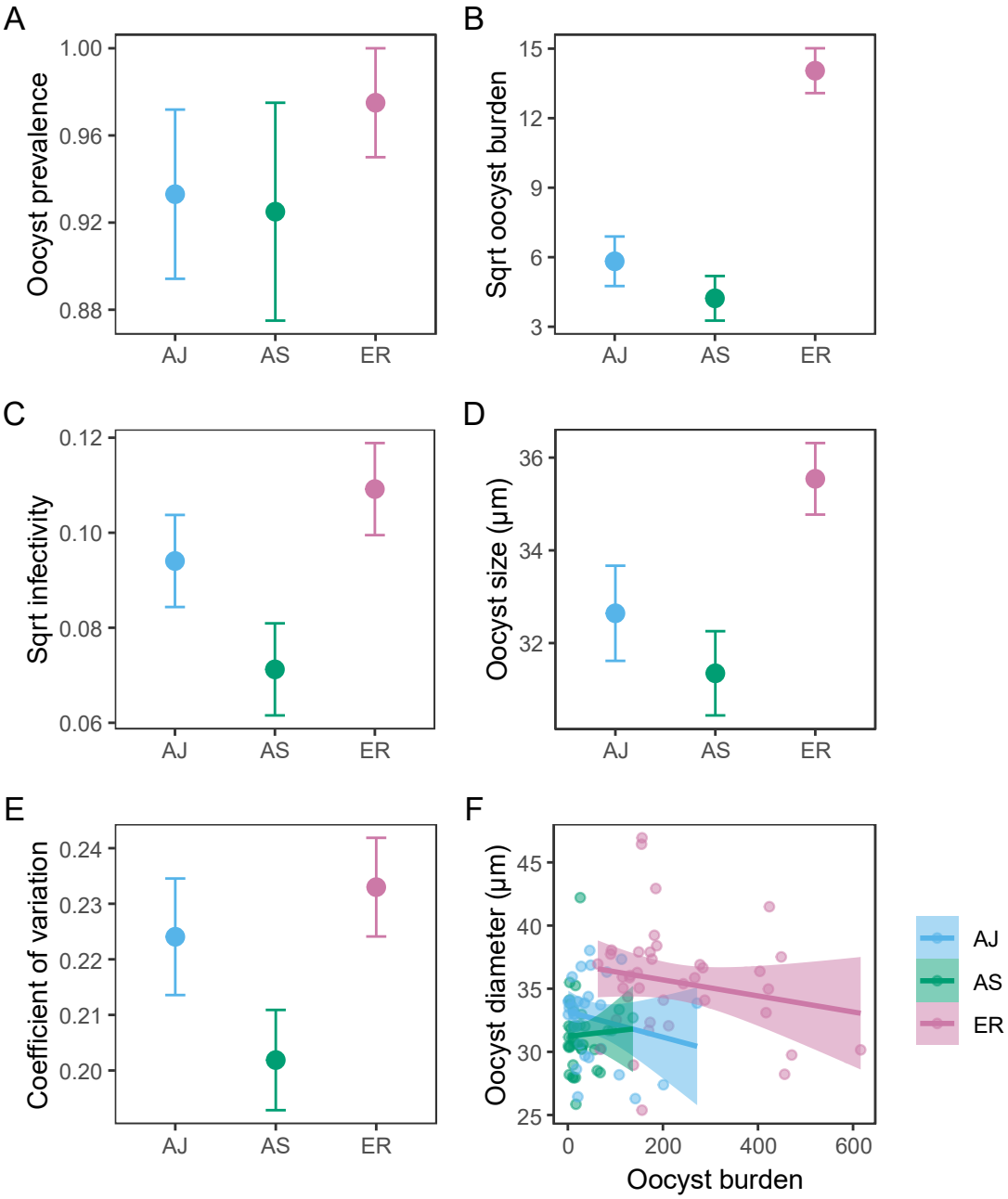

Figure 3.3. Oocyst development metrics for three *P. chabaudi* genotypes on day 7 post-infectious blood meal. Data presented are mean ± SEM for oocyst prevalence, defined as the proportion of infected mosquitoes (A), and predicted means ± SEM from minimised models are presented for oocyst burden (B), infectivity to mosquitoes (oocysts per gametocyte) (C), oocyst diameter (D) and oocyst size variation as measured by the coefficient of variation (E), and minimised model predictions per mosquito are presented for the correlation between oocyst burden and oocyst size, with lines and shading denoting best fit linear model estimates with 95% confidence intervals (F).

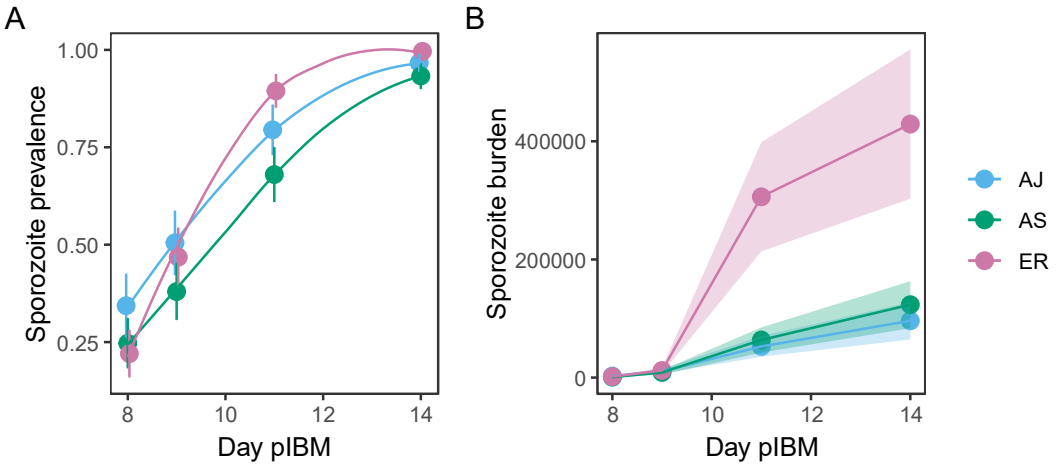

Figure 3.4. Sporozoite prevalence (A) and burden (B) across days 8-14 post-infectious blood meal (pIBM) for three *P. chabaudi* genotypes. Data presented are predicted means ± SEM from minimised models over four sampling time points in (A) and (B), plus a fitted logistic regression curve in (A).

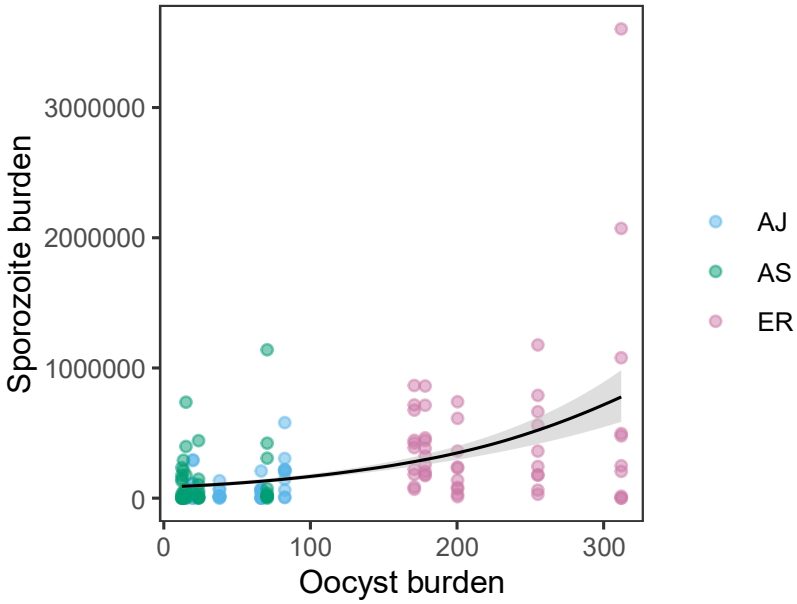

Figure 3.5. The correlation between oocyst and sporozoite burdens across three *P. chabaudi* genotypes. Lines and shading denote best fit negative binomial model estimates and standard error of predictions. Data points are individual mosquitoes.

3.4.3 Parasite dynamics during onward transmission differ across genotypes

To investigate whether genotypes differed in transmission from vector to host, we followed infections from mosquitoes to naïve mice. First, mice experienced a total sporozoite load from ER-infected mosquitoes that was 8-fold higher ($6.0x10^7 \pm 1.1x10^6$) than for AJ- or AS- (AJ: $9.4x10^5 \pm 5.3x10^5$, AS: $5.4x10^5 \pm 1.9x10^5$) exposed mice ($\chi^2_2=13.8$, $p<0.001$), which did not differ from each other (Figure 3.6A, Table 3.1). This was because individual mosquitoes infected with ER had 11-fold higher sporozoite burdens than mosquitoes infected with AS or AJ ($\chi^2_2=16.0$, $p<0.001$, Table 3.1).

There was no difference in the number of mice that became patent across genotypes ($\chi^2_2=2.55$, $p=0.28$); by day 6 post bite, all AS and ER mice (5/5 and 4/4 respectively) and 3/4 AJ mice had blood stage parasites. Furthermore, total sporozoite load did not influence the replication rate of parasites in the blood of mice for any genotype ($\chi^2_2=2.93$, $p=0.23$) or overall ($\chi^2_1=0.79$, $p=0.37$). While each genotype replicated at a similar day-to-day rate ($\chi^2_4=1.88$, $p=0.76$), initially increasing by 39-fold between days 4 and 6, then by 5-fold between days 6 and 8, asexual densities for AJ were 12-fold higher than for AS ($\chi^2_2=8.24$, $p=0.016$), but ER asexual densities did not differ from AJ or AS (Figure 3.6B, Table 3.1).

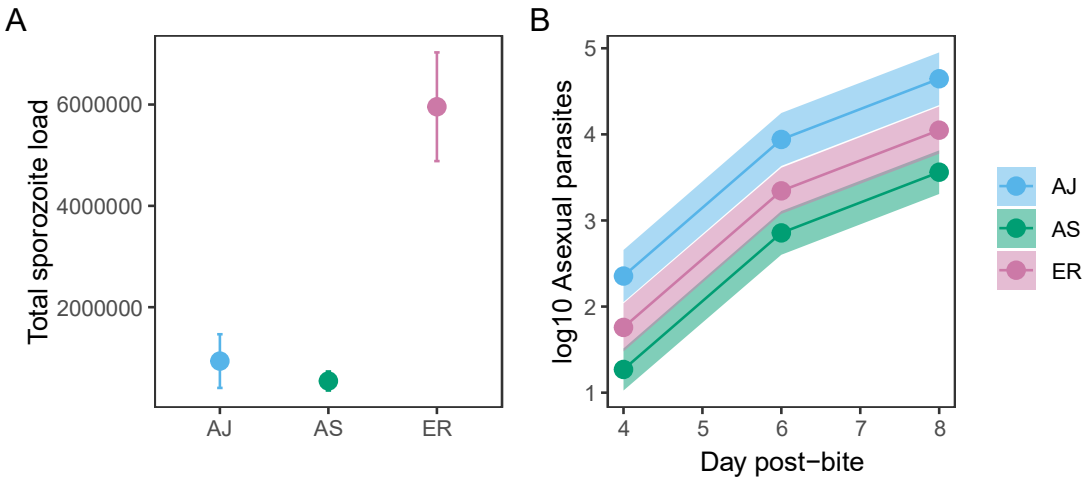

Figure 3.6. The impact of *P. chabaudi* genotype on total sporozoite load of mosquitoes used to infect naïve hosts (A) and subsequent asexual stage dynamics in the host. Data presented are means ± SEM for (A), and means ± SEM predicted from the minimised model (B).

Table 3.1. Post hoc pairwise comparisons between genotypes for transmission metrics. Significant p-values (<0.05) are highlighted in bold, and borderline p-values are italicised and underlined.

			Test statistic	*p-value*
Within host	Reticulocyte density	AS -- ER	*t* = 2.53	*0.07*
		AS -- AJ	*t* = -3.44	**0.01**
		AJ -- ER	*t* = -1.05	0.56
	Asexual density	AS -- ER	*t* = -4.14	**0.004**
		AS -- AJ	*t* = 4.62	**0.002**
		AJ -- ER	*t* = 0.71	0.76
	Gametocyte density	AS -- ER	*t* = -4.79	**<0.001**
		AS -- AJ	*t* = 0.33	0.94
		AJ -- ER	*t* = -5.42	**0.002**
Sporogony	Oocyst density	AS -- ER	*t* = -7.01	**<0.001**
		AS -- AJ	*t* = 1.07	0.55
		AJ -- ER	*t* = -5.51	**<0.001**
	Oocyst size	AS -- ER	*z* = -3.44	**0.002**
		AS -- AJ	*z* = 0.98	0.59
		AJ -- ER	*z* = -2.22	*0.07*
	Sporozoite prevalence (day 11 pIBM)	AS -- ER	*z* = -2.63	**0.02**
		AS -- AJ	*z* = 1.51	0.28
		AJ -- ER	*z* = -1.19	0.46
	Sporozoite burden (d11)	AS -- ER	*t* = -3.84	**<0.001**
		AS -- AJ	*t* = -0.62	0.81
		AJ -- ER	*t* = -4.38	**<0.001**
	Total sporozoite load	AS -- ER	*t* = -4.38	**0.005**
		AS -- AJ	*t* = 1.00	0.60
		AJ -- ER	*t* = -3.21	**0.03**
	Sporozoite burden in mosquitoes used for onwards transmission	AS -- ER	*t* = -4.84	**<0.001**
		AS -- AJ	*t* = -0.26	0.96
		AJ -- ER	*t* = -4.73	**<0.001**
Onwards transmission	Asexual density differences between genotypes	AS -- ER	*t* = -1.45	0.36
		AS -- AJ	*t* = 2.97	**0.04**
		AJ -- ER	*t* = 1.56	0.31

3.5 Discussion

We reveal that variation exists between different malaria genotypes in within-vector developmental (sporogonic) traits and in onward transmission to new hosts (Table 3.2). First, on the day of transmission to mosquitoes, mice infected with genotypes AJ and ER had higher asexual densities than AS, and ER produced more gametocytes than AJ or AS (Figure 3.2), but all genotypes infected mosquitoes to a similarly high prevalence (Figure 3.3). Second, ER's higher gametocyte density translated to more oocysts per midgut than for AS and AJ, though gametocytes for all genotypes were similarly infectious (Figure 3.3). Third, ER's oocysts grew fastest, followed by AJ, then AS, leading to ER achieving the highest sporozoite prevalence by day 11 pIBM (Figure 3.3,3.4). Yet, we did not observe a trade-off between the number and size of oocysts for any of the genotypes (Figure 3.3,3.4), nor did ER show higher variation in oocyst size within mosquito guts (Figure 3.3). Finally, we find a positive correlation between oocyst burden and sporozoite burden, indicating that ER's higher oocyst burden resulted in a greater sporozoite burden compared to AS and AJ (Figure 3.4,3.5). However, despite a higher sporozoite load, mice bitten by ER-infected mosquitoes were not more likely to become infected, nor did they have the highest parasite density in their blood (Figure 3.6). Instead, AJ reached the highest asexual densities during onward transmission, and AS the lowest, despite these two genotypes having similar trait values throughout sporogony, including the total sporozoite load that mice were exposed to (Figure 3.6).

Table 3.2. Summary of genotype differences during between-host transmission. Effect sizes are indicated when $p<0.05$ and for borderline trends (*BL;* $0.05<p<0.09$). *NS* = statistically non-significant.

		Summary of results
Within host	RBC density	NS
	Reticulocyte density	AS 1.3-1.6x > ER (*BL*) = AJ
	Asexual density	AJ = ER > 2.2x AS
	Gametocyte density	ER 3.0x > AS = AJ
Sporogony	Oocyst prevalence	*NS*, ~95%
	Oocyst density	ER 8x > AS = AJ
	Gametocyte infectivity	*BL*, where ER > AJ > AS
	Oocyst size	ER 9-13% > AJ (*BL*) = AS
	Variation in oocyst size	*BL*, where ER = AJ > AS

	Sporozoite prevalence dynamics	ER fastest but reach similar maxima
	Sporozoite density dynamics	ER > AS = AJ
	Oocyst productivity	*NS*
	Total sporozoite load	ER 8.0x > AS = AJ
Onwards transmission	Proportion mice infected	*NS* AS: 5/5, ER: 4/4, AJ: 3/4
	Asexual density dynamics	AJ 4-12x > ER (*NS*) = AS

Like previous studies, we found that genotypes differed in within-host traits. Genotypes ER and AJ had higher asexual parasite densities, suggesting faster asexual replication rates and higher within-host virulence than AS (Mackinnon and Read, 1999a). Virulence differences were not reflected in RBC densities, but this is likely to be because we used PHZ to stimulate gametocyte production early in infections, before significant anaemia occurs. Despite genotypes AJ and ER having similar asexual parasite densities, they invested differently in transmission, with AJ producing fewer gametocytes in line with previous studies (Pollitt et al., 2011b). In comparison, while AS had significantly lower asexual densities than AJ, their gametocyte densities were similar, suggesting higher investment in transmission. Investment into gametocytes is adjusted adaptively throughout infections in response to many factors (Pollitt et al., 2011b; Carter et al., 2013), including correlating positively with the availability of reticulocytes (Birget et al., 2017). That AS invested more in gametocytes than AJ is in keeping with its hosts harbouring more reticulocytes and that it is more sensitive to reticulocyte density (Birget et al., 2017). While differences in gametocyte densities did not impact oocyst prevalence in mosquitoes, which has also been observed in previous studies (Guissou et al., 2023), ER's higher gametocyte density did result in more oocysts per mosquito. Although per gametocyte infectivity did not significantly differ between genotypes, further studies with greater statistical power are needed to investigate the trend that ER gametocytes may be more infective. Additionally, mosquito factors, such as immune system responses, may vary due to differences in immune evasion strategies across parasite genotypes (Smith and Barillas-Mury, 2016). Understanding how these factors interact is needed to fully explain the higher oocyst burden in ER-infected mosquitoes. However, overall, our findings suggest that the quantity (i.e. density) and quality (i.e. infectivity) of gametocytes do not trade off against each other.

Oocyst burdens showed the same genotype-specific differences as previous studies; ER-infected mosquitoes had higher burdens than AS- and AJ-infected mosquitoes which did not differ (Ferguson et al., 2003a). However, burdens in our study may have been elevated by the high gametocyte densities generated by using PHZ. This may have also eroded the genotype-specific differences in oocyst prevalence observed in previous studies (Ferguson et al., 2003a). Our data also suggest the burden of oocysts does not trade off against their productivity (i.e. sporozoites per oocyst) or size (i.e. growth of oocysts) across any genotypes. This, and the lack of genotype differences in variability in oocyst size, are surprising because oocysts in high density infections are expected to cause competition between oocysts for resources (Sinden and Strong, 1978; Pollitt et al., 2013; Habtewold et al., 2021). Instead, we find that genotype ER had the most and largest oocysts on day 7 pIBM. Larger oocyst size, along with genotype ER having the highest sporozoite prevalence by day 11 pIBM, suggests that ER develops at the fastest rate (Werling et al., 2019; Shaw et al., 2020; Habtewold et al., 2021; Kanatani et al., 2024), accumulating sporozoites sooner in the salivary glands (Shaw et al., 2020). The EIP of malaria is surprisingly long relative to the average lifespan of mosquitoes (Clements and Paterson, 1981; Siria et al., 2022; Carrillo-Bustamante et al., 2023), so small reductions in the EIP can dramatically increase transmission. While a long EIP is thought to have evolved to maximise the exploitation of vector resources (Carrillo-Bustamante et al., 2023), we also find that ER (with a shorter EIP) produces the highest number of sporozoites because of its high oocyst burden. Assuming that all genotypes are well-adapted, the lack of trade-offs between oocyst metrics within genotypes is unlikely to be explained by all genotypes having plentiful resources for development, because genotype-specific differences in oocyst burdens, oocyst size and subsequent sporozoite burdens suggest some underlying trade-offs. Therefore, perhaps genotype ER pays an alternative cost from producing large and productive oocysts quickly, such as a trade-off between quantity/development speed vs. quality (i.e. infectivity to the vertebrate host) of sporozoites.

Indeed, our data suggest that sporozoite infectivity and/or inoculum size differs across genotypes. The higher sporozoite burden of ER-infected mosquitoes did not increase the probability of onward transmission to mice, nor did it result in higher densities of asexual blood stages. This is unexpected, given that previous studies suggest higher sporozoite burdens result in more infected hosts (Churcher et al., 2017; Aleshnick et al., 2020; Kanatani et al., 2024). While the number of sporozoites in the salivary glands and the number inoculated during blood feeding correlate across species and

within single isolates, whether intraspecific variation exists is unknown (Guissou et al., 2021; Oke et al., 2023; Andolina et al., 2024; Kanatani et al., 2024). Perhaps the high number of ER sporozoites in the salivary glands causes crowding, which reduces the number inoculated compared to AS and AJ. Fewer inoculated sporozoites could explain why ER fails to produce as many asexual stages in the blood as AJ, but ER sporozoites might also (or alternatively) be of low quality, struggling to evade skin defences or invade liver cells. Alternatively, ER sporozoites may efficiently infect hosts and complete liver phase development and its lower asexual stage density is due to its blood stages being more vulnerable to immune killing or poorer at invading red blood cells. However, this is unlikely because ER is very good at exploiting the host (it reaches as high asexual parasite densities as AJ later in infections (Mackinnon and Read, 1999a; Mackinnon et al., 2005) (Figure 3.2C). Instead, we predict that genotype ER produces lower quality sporozoites than AS and AJ because its fast development limits lipid acquisition by oocysts. Restricting lipids reduces sporozoite infectivity via altered metabolic activity (Costa et al., 2018) and may reduce sporozoite lifespan by accelerating senescence (Van Schuijlenburg et al., 2024). Because high oocyst burdens can reduce mosquito lifespan (Pollitt et al., 2013), ER might develop faster at the expense of sporozoite quality to ensure sporozoites reach the glands before their vector dies. If so, developmental speed may be ultimately constrained by the ability of mosquitoes to acquire resources, because fast development could cause overexploitation, further decreasing vector lifespan. The costs of fast development are also offset in mixed infections. Mixed infections accelerate vector mortality (Ferguson and Read, 2002), making it advantageous to be the first genotype to reach the salivary glands. Thus, genotype ER might have been more likely to experience mixed infections than AJ and AS during its evolutionary history. While other studies have observed a positive correlation between sporozoite burden and a short EIP (Andolina et al., 2024), further work is needed to elucidate which aspects of sporozoite quality can be affected by fast development.

Parasite genotypes that exhibit lower oocyst burdens (like AS and AJ) may maximise fitness by being able to accumulate more resources by spreading exploitation of the vector over a longer period (i.e. spanning additional blood meals) (Carrillo-Bustamante et al., 2023) and/or because there are more resources per oocyst, both resulting in high quality sporozoites (Shaw et al., 2022) and minimising premature vector death. Our results suggest the latter is the case for AJ, which had the second-fastest EIP and fewer sporozoites than ER, but produced similar numbers of asexual stage parasites. In comparison, genotype AS had the slowest development and

produced similar numbers of sporozoites to AJ, but had lower asexual densities in the blood despite similar replication rates in the first cycles of infection. Perhaps genotype AJ is better at exploiting mosquito resources than AS (it exhibits higher virulence in the host), and AS produces lower quality sporozoites because it is more resource constrained. Additionally, if the marginal trend for AS's lower variability in oocyst size is supported in further studies, this suggests that AS could be 'waiting' for resources to improve sporozoite quality, because an additional blood meal can enhance infectivity (Costa et al., 2018; Habtewold et al., 2021; Carrillo-Bustamante et al., 2023). Therefore, future work should assess how genotypes respond to variable resources; for example, additional blood meals may improve the quality of AS's sporozoites compared to other genotypes (i.e. genotype-by-environment interactions) (Habtewold et al., 2021; Carrillo-Bustamante et al., 2023). To clarify whether the differences in asexual density in the blood stage of infection are due to parasite traits expressed in the vector, host, or both, future work should investigate genotype differences in sporozoite inoculum (Guissou et al., 2021; Oke et al., 2023), sporozoite quality in vitro (Beyer et al., 2021) and in the skin (Hopp et al., 2015), and liver parasite burdens, as well as whether changes in the within-vector environment alters these traits.

We have demonstrated that within-vector traits differ across genotypes and have hypothesised that may be due to genotypes adopting different strategies to optimise transmission. How might this variation influence evolution in response to vector control and climate change induced changes to mosquitoes? For example, insecticide exposure (Viana et al., 2016) reduces vector lifespan so faster developing genotypes have a fitness advantage because they are more likely to complete the life cycle. However, insecticide resistance reduces nutritional resources within the vector (Rivero et al., 2011), so avirulent genotypes are fitter because they are less likely to overexploit the mosquito (Ferguson and Read, 2002). However, faster developing genotypes could be favoured if fewer resources also reduces mosquito lifespan (see Chapter 4). In addition, within-vector development can influence disease dynamics within the host (Mackinnon et al., 2005; Spence et al., 2013). For example, if genotypes with a short EIP are also more virulent in the host, insecticides could unintentionally select for parasites causing more severe clinical disease by reducing vector lifespan. Our findings also demonstrate that the commonly-used one vector genotype-one parasite genotype combination is unlikely to reflect the variation in phenotypes observed across a parasite population (Lambrechts et al., 2005; Molina-Cruz et al., 2012). Differences in the within-vector environment such as nutritional

resources and age are also likely to be sources of variation in parasite transmission traits. Thus, future studies should examine multiple parasite genotypes transmitting via mosquitoes with different genotypes and phenotypes, but it is very challenging to make such large scale experiments tractable. Nonetheless, knowledge about heritable genetic variation in parasite phenotypes, the extent of plasticity, and how these interact are necessary to predict parasite evolution in response to interventions that interfere with transmission (Schneider and Reece, 2021; Oke et al., 2022).

Chapter 4. Plasticity in malaria parasite development: mosquito resources influence vector-to-host transmission potential

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4.1 Abstract

Parasites rely on exploiting resources from their hosts and vectors for survival and transmission. This includes nutritional resources, which vary in availability between different hosts and change during infections. For malaria (*Plasmodium*) parasites, sexual reproduction (sporogony) and subsequent development of oocysts, which produce sporozoites infectious to the vertebrate host, occurs in the mosquito vector. Mosquitoes in the field exhibit diversity in the amount and type of food they acquire, directly impacting the nutrients available for the replication and development of parasites. While the rate of parasite transmission from vector to host is influenced by the nutritional state of mosquitoes, whether this is due to resource limitation mediating parasite development and productivity is poorly understood. We use the rodent model parasite *P. chabaudi* and the vector *Anopheles stephensi* to ask how variation in the amount of sugar and blood provided to malaria-infected mosquitoes affects the potential for parasites to transmit from vector to host. We show that parasites in well-resourced mosquitoes reach a larger oocyst size earlier in development, suggesting faster growth, and have a 1.7-fold higher sporozoite burden than parasites whose vectors only receive sugar. However, this increase in productivity is only partly explained by oocyst development, suggesting that resource availability also impacts the ability of sporozoites to reach the salivary glands. This challenges the assumption of a simple relationship between the number or size of oocysts and onward transmission potential. Furthermore, our findings suggest malaria parasites may actively adjust oocyst growth rate to best exploit nutritional resources; while parasites in low-resourced mosquitoes exhibited a reduction in oocyst burden during sporogony, the remaining oocysts developed more rapidly in the later stages of oocyst development, catching up to reach a similar size to those in well-resourced

mosquitoes. Understanding the impacts of resource availability for malaria transmission is urgent given that parasites encounter increasingly variable vectors as consequences of climate change and vector control tools.

4.2 Introduction

Parasites by definition rely on hosts, exploiting them for food and shelter (Rózsa and Garay, 2023). Many parasite species, including vector-transmitted parasites, have multi-host lifecycles, where different life stages are found in phylogenetically diverse definitive and intermediate host species (Webster et al., 2017). These parasites are exposed to extensive variation in the within-host/vector environmental conditions they experience, and so, adapt to exploit environments which provide different types and amounts of resources. For example, malaria parasites (*Plasmodium*) exploit nutritional resources in the blood of their vertebrate host, but also must be able to successfully utilise resources present in the gut and haemolymph of their insect vector (Lefevre et al., 2017). In addition, parasites face variation in how well-resourced individual con-specific hosts/vectors are, and in how nutritional resources vary during the course of infections within hosts/vectors. For example, resource availability in mosquito vectors can vary across larval breeding habitats which subsequently affects adult fitness (Takken et al., 2013; Shapiro et al., 2016), and for malaria parasites, the density of red blood cells (RBC) available within the vertebrate host reduces as parasite density increases, and the resulting anaemia leads to an alteration in the RBC age structure as the host recovers (Birget et al., 2017). Other causes of variation between individual hosts/vectors include immune status, infection with other parasites, age, sex and reproductive status, which can also alter the nutritional resources available for parasites to exploit. For example, vectors show age-specific changes in foraging (Alto et al., 2003; Cator et al., 2020), and co-infection of multiple parasite strains causes competition for limited resources within the vertebrate host (Pollitt et al., 2011b).

The nutritional resources available within vectors impacts parasite transmission by influencing multiple parameters of vectorial capacity (i.e. the efficiency of vector-borne disease transmission under natural conditions) (Takken et al., 2013; Shapiro et al., 2016; Almire et al., 2021). Adult female mosquitoes rely on both blood and sugar sources for nutrition during their lifespan; blood provides a nutritional boost of proteins and lipids which are necessary for egg production, and sugar sources provide the majority of energy for meeting the mosquito's metabolic needs, including powering

processes such as flight (Foster, 1995). Climatic variation (Gu et al., 2011; Stone et al., 2012), season, and access to vertebrate hosts (which is reduced by bed net use (Killeen et al., 2007)) generate variation in blood and sugar availability, impacting vector behaviour, lifespan and fecundity (Fernandes and Briegel, 2005; Gary and Foster, 2006; Ma and Roitberg, 2008; Lyimo et al., 2012; Dieng et al., 2015; Hagan et al., 2018; Barredo and Degennaro, 2020; Yan et al., 2021; Vantaux et al., 2023). Furthermore, the type of plant sugar and blood meal sources used by mosquitoes determines their longevity, biting behaviour and susceptibility to malaria infection (Hien et al., 2016; Emami et al., 2017; Ebrahimi et al., 2018; Vantaux et al., 2023). Thus, the availability and quality of nutritional resources available to mosquitoes affects vectorial capacity via vector population dynamics, susceptibility to infection, and biting rates. However, whether and how nutritional resources mediate transmission via additional direct effects on parasite development and productivity within vectors is poorly understood.

Malaria parasites rely on nutrients from the vector's blood and sugar meals throughout sporogony. Sporogony involves gametogenesis and fertilisation within the mosquito midgut, traversal of the mosquito midgut wall to form oocysts, and extensive growth and replication within oocysts to produce tens of thousands of vertebrate-host-infectious sporozoites, which must egress from oocysts and migrate to the salivary glands (Beier, 1998). Glucose is required to power microgamete motility during mating (Talman et al., 2014), and mosquito derived-lipids and amino acids are required for sporozoite production (Atella et al., 2009; Costa et al., 2018; Shaw et al., 2020; Kwon et al., 2021). Thus, reducing the amount of lipids available to oocysts lowers the quantity of sporozoites as well as their ability to infect a vertebrate host (Costa et al., 2018), and feeding already-infected mosquitoes an additional blood meal can speed up oocyst development (Shaw et al., 2020; Kwon et al., 2021). However, the overall impacts of nutritional resources on transmission, and to what extent parasites can optimise their amount and schedule of resource exploitation, are unknown.

Theory for the evolution of life history traits predicts that while natural selection acts to maximise fitness, expression of traits is limited by trade-offs and constraints commonly caused by limited resources available for growth, maintenance and reproduction (Stearns, 1989). Applying life history theory to parasites has revealed sophisticated strategies deployed within the host (Reece et al., 2009; Schneider and Reece, 2021), and could help to understand parasite strategies within the vector (Carrillo-Bustamante et al., 2023). A key concept is that each highly genetically related genotype of single-celled malaria parasites within an infection can be viewed as an

organism from the perspective of natural selection (Gardner and Grafen, 2009; Schneider and Reece, 2021), adopting strategies that are collectively beneficial for the genotype. For example, in the vertebrate host, the rodent malaria *P. chabaudi* plastically alters the sex ratio of sexual transmission stage parasites (gametocytes) (Reece et al., 2008) and the proportion of asexual parasites committing to form gametocytes (Schneider et al., 2018a). Specifically, parasite genotypes adjust these traits during infections, and between different hosts, in manners that maintain fitness across different environmental conditions (Pigliucci, 2005), such as variable degrees of competition with co-infecting genotypes and the availability of red blood cell resources (De Roode et al., 2005; Bell et al., 2006; Birget et al., 2017).

Here, we apply life history theory to explain parasite strategies in the vector, and how they may maximise fitness (i.e. between-host transmission). For example, a common trade-off is offspring quality vs quantity, and parasites may also face this trade-off during sporozoite production, the strength of which will vary dependent on resource availability. For example, under nutrient-limiting conditions, a small number of oocysts could continue growing, to ensure completion of sporogony for a small number of sporozoites rather than producing more lower quality sporozoites or risk not completing sporogony at all (Habtewold et al., 2021). Additionally, the length of time it takes for parasites to complete sporogony (the extrinsic incubation period, EIP) is dependent on mosquito resources (Shaw et al., 2020). If resources are limiting, faster development may occur at the expense of quality and/or quantity of sporozoites, or alternatively may be extended to wait for additional incoming resources (Carrillo-Bustamante et al., 2023). Conversely, excess resources may allow parasites to develop unhindered, producing more high quality sporozoites at a fast rate. Following these scenarios, parasites in medium-resourced mosquitoes should adopt intermediate strategies, constrained to developing slower than in well-resourced mosquitoes, but producing more/better sporozoites than low-resourced mosquitoes. How parasites respond to resource availability will also depend on whether, and how, parasites sense resource supply, and whether vector death is imminent or if the vector will survive long enough to forage for more resources.

While previous studies suggest parasites capitalise on an increase in resource availability by speeding up sporogony (Shaw et al., 2020), it is unknown whether parasite development and productivity are limited in poorly-resourced mosquitoes. By perturbing mosquito nutritional status after infection with *P. chabaudi*, we investigate how parasites respond to both an increase and decrease in the availability of within-vector resources. We provide mosquitoes with (i) low fructose, representing vectors

with limited access to all resources, (ii) high fructose, representing vectors with limited access to blood meals but plentiful resources for their survival, or (iii) high fructose plus an additional blood meal, representing well-fed vectors with access to both types of resources. We hypothesise that if parasites can detect changes in resource availability and/or vector condition, they will adopt different optimal development strategies to maximise transmission, including changes to sporozoite quantity, quality and/or developmental speed (i.e. the EIP). Overall, we demonstrate that parasite development during sporogony is plastic and propose hypotheses for future studies to differentiate whether this is due to constraints or whether parasites are adopting adaptive (fitness-maximising) strategies. We also explore implications for transmission potential because well-resourced mosquitoes are likely to have higher vectorial capacity.

4.3 Materials and methods

We performed two experiments to test how nutritional resources garnered by mosquitoes affect malaria parasite development and onward transmission potential. First, we confirmed the extent to which different food treatments perturbed mosquito physiological condition by comparing survival rates and nutritional status in uninfected mosquitoes. Second, we infected mosquitoes with *P. chabaudi* and subsequently perturbed food treatments to quantify the impact on oocyst development and sporozoite burden.

4.3.1 Vectors, hosts, and parasites

We used the highly tractable *Anopheles stephensi*-*P. chabaudi*-lab mouse model system, which closely reflects the within-host behaviours and within-vector dynamics of the most virulent human malaria parasite, *P. falciparum*, in its most common (*An. gambiae* complex) vectors (Spence et al., 2012; Schneider and Reece, 2021; Shaw et al., 2022). We housed adult *An. stephensi* SD500 mosquitoes at 26°C, 70% relative humidity on a 12L: 12D cycle and provided them with *ad libitum* access to 8% fructose solution following emergence. Three days post-pupation, we transferred adult female mosquitoes to experimental cages. In both experiments, we provided mosquitoes with an initial blood meal at 5-7 days post-pupation. Prior to the initial blood meal, we starved mosquitoes overnight by providing water only. On day 1 post-blood meal (pBM), we removed unfed females from cages, and on day 3 pBM we provided an

oviposition site (a small petri dish lined with filter paper, filled with water) in each cage to give mosquitoes the opportunity to lay eggs.

For all uninfected blood meals in the first experiment, and additional blood meals in the second experiment, we used 8-10 week old male and female *Per1/2*-null (on a C57Bl/6 J background) clock disrupted mice (O'Donnell et al., 2020) housed under standard 12L:12D conditions with *ad libitum* access to food and drinking water. When housed in 12L:12D conditions, *Per1/2*-null mice exhibit a wildtype rhythmic phenotype (Bae et al., 2001), and were excess from a breeding colony to ethically reduce the number of mice specifically bred for the present study (Prescott and Lidster, 2017). To infect mosquitoes with malaria in the second experiment during their initial blood meal, we allowed female mosquitoes to blood feed on mice infected with *P. chabaudi* (genotype ER, passage number A17). Mice were 10 week old C57Bl/6 J male mice given *ad libitum* access to food and drinking water (supplemented with 0.05% para-aminobenzoic acid to aid parasite development (Jacobs, 1964)). We pre-treated mice with 125mg/kg phenylhydrazine (PHZ) prior to infection to enhance gametocyte production. Three days after PHZ treatment, we infected mice with $1x10^7$ *P. chabaudi* ring stage parasitised red blood cells by intraperitoneal (IP) injection. Transmissions occurred on day 5 post-infection. On day 4 post-infection, we took blood samples from all mice for quantification of gametocytes by microscopy, selecting those with the highest gametocytemia for transmission feeds. Prior to donating a blood meal, we anaesthetised mice (65mg/kg ketamine hydrochloride and 0.85mg/kg medetomidine in PBS) and placed each mouse on a cage of mosquitoes for 20 minutes.

4.3.2 Experiment 1: Resource perturbation in uninfected mosquitoes

To confirm that varying the availability of nutritional resources successfully perturbed mosquito condition, we assessed the impact of four feeding treatments on mosquito nutritional status (lipid, glycogen and total sugars content) and lifespan without the confounding effects of malaria infection (Figure 4.1A). We first allowed a cage of female mosquitoes (n=1000) to take an initial blood meal 5-7 days post-pupation from four mice, then placed them on *ad libitum* 8% fructose solution. Three days later (day 3 pBM, to follow a gonotrophic cycle), we randomly transferred n=40 mosquitoes to each of 24 paper pots with mesh lids and oviposition sites. The following day (day 4 pBM), we randomly allocated six pots to each of four treatments, which involved *ad libitum* access to either: (i) 0.08% fructose (0.08% fruc), (ii) 0.8% fructose (0.8% fruc),

(iii) 8% fructose (8% fruc) and (iv) 8% fructose and an additional blood meal on day 4 pBM (8% fruc + bm).

From day 4 pBM we tracked mosquito survival by recording daily deaths until day 35 pBM (n=5 pots per treatment group). On day 9 pBM, we collected n=16 mosquitoes from the sixth pot (n=1 pot per treatment group), and froze them at -20°C before undertaking lipid, glycogen and total sugar analyses following modified Van Handel protocols (Van Handel, 1985a, 1985b; Van Handel and Day, 1988). In brief, we removed a wing from each mosquito to quantify body size, and lysed individual mosquitoes in 100µl 2% sodium sulphate and added 750µl of 1:2 chloroform:methanol. We centrifuged samples at 17949x*g* for 3 minutes; the supernatant was collected for lipid and total sugars analysis, and the precipitate was used for glycogen analysis. We were unable to collect data on lipids and total sugars from two mosquitoes. We conducted lipid analysis following Meuti *et al* (Meuti et al., 2015) with minor modifications: 250µl supernatant was used, and 80µl of sample was added to 96-well plates and we measured absorbance at 525nm and 490nm on a microplate reader (Varioskan Flash, ThermoFisher Scientific). We carried out glycogen analysis by adding 200µl anthrone reagent to the precipitate, and heating to 90°C for 15 minutes. After cooling at 4°C for 5 minutes, we added 40µl to a 96-well plate containing 40µl anthrone reagent and absorbance was measured at 625nm and 555nm on a microplate reader. Total sugars analysis followed the same protocol to glycogen with minor differences: we heated 125µl of supernatant at 90°C until the solvent evaporated, and then added 200µl anthrone reagent. After heating and cooling as above, we added 80µl to 96-well plates before measuring absorbance at the same wavelengths.

4.3.3 Experiment 2: Resource perturbation in infected mosquitoes

To investigate how perturbations of nutritional status of mosquito vectors affect sporogony, we fed all mosquitoes an infected blood meal, and then assessed the impact of three feeding treatments on: (i) oocyst prevalence (the proportion of mosquitoes infected with oocysts), (ii) oocyst burden (the number of oocysts per gut in infected mosquitoes), (iii) oocyst size (a proxy for growth rate), (iv) sporozoite prevalence (the proportion of mosquitoes with detectable sporozoites), and (v) sporozoite burden (the number of sporozoites in individual mosquitoes) (Figure 4.1B). To infect mosquitoes, we anaesthetised infected mice (n=30) and exposed each mouse to a pot of mosquitoes (n=12 pots per treatment group, n=80 mosquitoes per

pot) for 20 min, with the exception of the six mice with the highest gametocyte loads, which were simultaneously exposed to pairs of pots. We took blood samples from mice at the point of transmissions to confirm gametocyte numbers by quantitative reverse-transcriptase PCR (RT-qPCR) (Schneider et al., 2018a).

Pots of mosquitoes were randomly allocated to treatment groups on day 1 post-infectious blood meal (pIBM): (i) low fructose (low fruc; 0.08% fructose for 6 days, increased to 0.8% fructose thereafter), (ii) 8% fructose (8% fruc) or (iii) 8% fructose plus an additional blood meal on day 4 pIBM (8% fruc + bm). Despite pilot data revealing 0.08% fructose is sufficient for naïve mosquitoes to survive the experimental period, this proved not be the case for infected mosquitoes. Because deaths within the first few days of infection were unexpectedly high in the low fructose group, we ensured enough mosquitoes survived for the duration of data collection by increasing fructose concentration to 0.8% from day 6 pIBM. By perturbing the diets of mosquitoes once infected, we avoided any impacts of altering mosquitoes prior to infection in manners that could confound impacts on parasite development by affecting mosquito uptake of infectious blood and susceptibility.

On days 7 and 10 pIBM, we sampled mosquitoes (n=6 per pot per time point) to determine oocyst prevalence, burden, and size. On day 10, we collected mosquitoes from only 6 pots in the low fructose group due to the low survival described above. To visualise oocysts, we anaesthetised mosquitoes on ice, and dissected midguts in a drop of phosphate buffered saline (PBS). Dissected midguts were stained in 0.25% mercurochrome and photographed at 40x magnification using a light microscope on a single focal plane. Using ImageJ (Schneider et al., 2012a), we placed a grid over each midgut photograph and used this to count total oocysts, and measure the area of up to 30 oocysts per gut, starting at the central grid square in the midgut. Measurements were calibrated using a photograph of a micrometer taken at the same magnification. We also used these measurements to calculate a metric we termed oocyst coverage, that accounts for changes in both the number and size of oocysts as a proxy for the total biomass of each infection (oocyst coverage = mean oocyst volume multiplied by burden per mosquito gut). On days 11 and 14 pIBM, we sampled mosquitoes (n=8 per pot) to determine sporozoite prevalence and burden over time. Mosquitoes were anaesthetised on ice, and bisected following Foley *et al* (Foley et al., 2012). We stored head/thorax samples at -20°C until DNA extraction and quantification by qPCR (Schneider et al., 2018b).

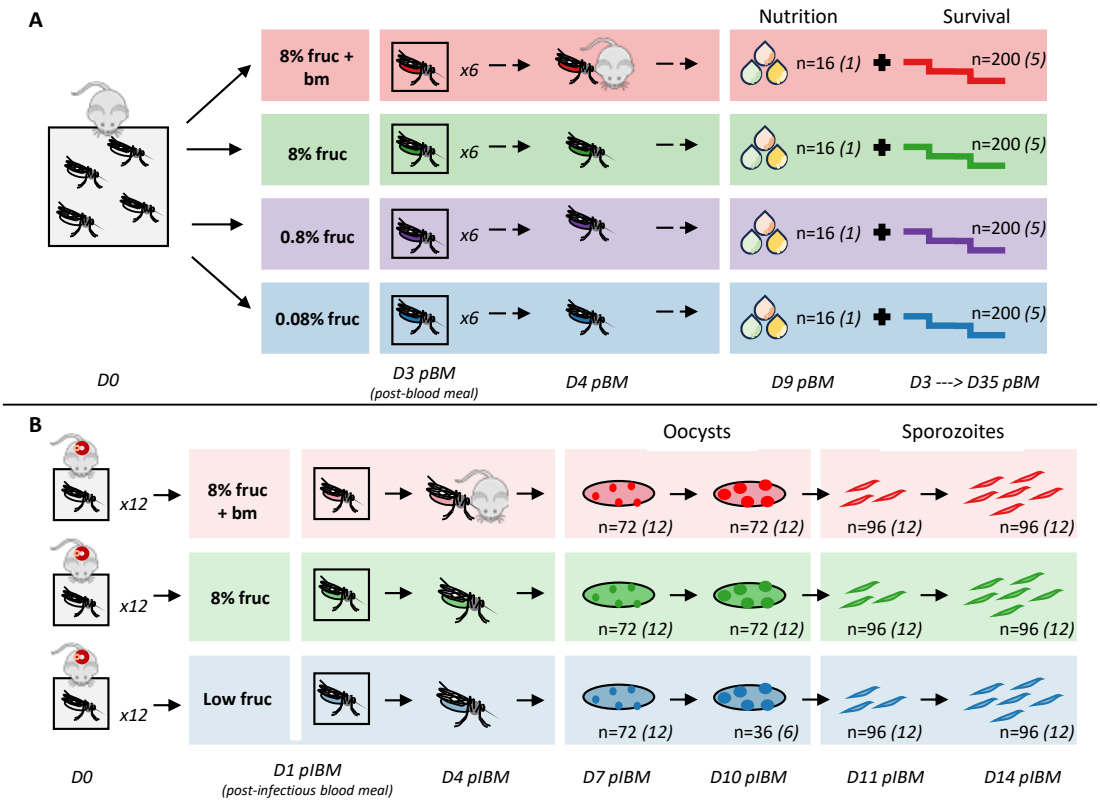

Figure 4.1. Experimental design and sampling for investigating the effect of resource availability on nutritional status (lipids, glycogen, total sugars) and survival in uninfected mosquitoes (A) and parasite development within infected mosquitoes (B). Sample sizes (n) refer to the total number of individual mosquitoes collected for each sampling point across replicate pots (denoted by number in italics).

4.3.4 Nucleic acid extraction and quantification of parasites

To quantify gametocyte densities in mice used to infect mosquitoes, we extracted RNA from 10µl blood samples following Schneider *et al* (Schneider et al., 2018a), and used an RT-qPCR assay targeting the sexual stage-specific expressed gene *PSOP1* (PCHAS_0620900) (Wargo et al., 2006). To quantify sporozoite burden and prevalence, we extracted DNA from mosquito head/thorax samples following the CTAB-based phenol-chloroform extraction method from Chen *et al* (Chen et al., 2010) with minor modifications as described in Schneider *et al* (Schneider et al., 2018b). We eluted extracted DNA in 30µl of water, and froze at -20°C until use. A qPCR targeting a region of the 18S rRNA gene (Bell et al., 2009) was used to quantify salivary gland sporozoites. Quantification of parasite genomes was determined by comparing threshold cycle (Ct) against a standard curve, generated from DNA extracted from microscopy-quantified blood stage parasites of *P. chabaudi* genotype ER.

4.3.5 Statistical analysis

We used R v. 4.1.3 for data analysis. We analysed nutritional content (lipid, glycogen, total sugars) of mosquitoes using linear models with feeding treatment as a main effect. We corrected for body size by dividing nutritional content by wing length, and then multiplying by the mean wing length across all mosquitoes. To estimate how feeding treatment affected survival in uninfected mosquitoes, we used Kaplan-Meier curves (*survminer*, (Kassambara et al., 2021)) and a cox proportional hazards model with mosquito pot as a cluster to account for non-independent measures within mosquito pots (*coxme*, (Therneau, 2022)). We confirmed that pots of mosquitoes across feeding treatments were provided with similar gametocytaemias using a linear model (feeding treatment: $F_{2, 33}$=0.56, p = 0.58).

For all subsequent models investigating parasite metrics, we included mouse as a random effect to account for pots fed simultaneously on the same mouse, and we added a random effect of mosquito pot or a nested random effect of mosquito identity within mosquito pot to account for non-independent measures within mosquito pots and mosquito guts respectively. We tested the impact of feeding treatment, day pIBM and their interaction on oocyst and sporozoite prevalences, using binomial generalised linear mixed models (glmm). We used linear mixed models (lmm) to investigate how feeding treatment, day pIBM and their interaction affected (i) oocyst burden of infected mosquitoes, (ii) oocyst coverage, (iii) oocyst diameter, (iv) oocyst size variation within a gut (coefficient of variation: standard deviation of oocyst diameter per gut divided by mean oocyst diameter per gut), and (v) sporozoite burden. We also used a lmm to investigate how feeding treatment affected the correlation between oocyst diameter and oocyst burden on day 7 pIBM. Similar lmms were used to test for correlations between sporozoite burden on day 14 pIBM and pooled means per pot for (i) oocyst diameter, (ii) square root oocyst coverage, and (iii) square root oocyst burden on day 10 pIBM, and whether these varied by feeding treatment.

We minimised all models using likelihood ratio tests, and corrected Akaike information criterion (AICc) for non-nested models, and tested for conformation to model assumptions using *DHARMa* (Hartig, 2022), or *easystats* (Makowski et al., 2020) for models with nested random effects. Nutrition, sporozoite burden, oocyst burden and oocyst coverage data were all square-root transformed to meet assumptions of normality and homogeneity of variance. We present predicted means from the best fitting minimised models (predict() function, *lme4*, (Bates et al., 2015)), and estimated SEMs from parametric bootstrapping (bootMer function). We used model predictions to fit linear regression curves. We conducted post hoc pairwise comparisons using

the Tukey method with the *emmeans* package (Lenth, 2023) for minimised models containing a significant effect.

4.3.6 Ethics approval statement

All animal procedures complied with the UK Home Office regulations (Animals Scientific Procedures Act 1986; SI 2012/3039; PPL PP8390310) and were approved by the University of Edinburgh.

4.4 Results

4.4.1 Resources affect mosquito nutrition and survival

We used uninfected mosquitoes to verify that varying the concentration of fructose in sugar meals and an additional blood meal affected lipid ($F_{3, 58}$=43.9, $p < 0.001$), glycogen ($F_{3, 60}$=10.7, $p < 0.001$) and total sugars ($F_{3, 58}$=91.8, $p < 0.001$) content (Figure 4.2). Mosquitoes fed 8% fructose contained 357±61.8µg lipids each, which was approx. 10-fold higher than mosquitoes fed 0.08% fructose (38.6±7.80µg), but 1.7-fold lower than when an additional blood was given (594±65.6µg) (Figure 4.2A, Table 4.1). Similarly, for total sugars, mosquitoes on the lowest fructose diets contained 44.1±15.7µg each, 20-fold lower than those fed on 8% fructose (896±56.5µg). However, an additional blood meal only slightly increased total sugar content to 1065±132µg compared to 8% fructose (Figure 4.2B, Table 4.1). In contrast, glycogen stores were similar across mosquitoes fed 0.8% or more fructose (204-262µg), but were approximately 2.7-fold lower in mosquitoes fed 0.08% fructose (85.5±9.81µg) (Figure 4.2C, Table 4.1).

Mosquito survival was also affected by feeding treatment. Compared to mosquitoes fed 8% fructose, an additional blood meal increased lifespan (HR: 0.41, 95% CI 20.6-44.8, z = -6.17, $p < 0.001$), whereas those given 0.08% fructose had the fastest mortality rate (HR: 30.4, 95% CI 0.70-1.36, z = 17.2, $p < 0.001$), with a median survival of 11 days (95% CI 11-12). However, there was no difference between groups given intermediate levels (0.8% and 8%) of fructose (HR: 0.98, 95% CI 0.32-0.55, z = -0.135, p = 0.892), which had median survivals of 32 (95% CI 30-34) and 33 (95% CI 31-34) days respectively (Figure 4.3). Note, too few mosquitoes died within the 35 day monitoring window to accurately predict median survival for mosquitoes given an additional blood meal.

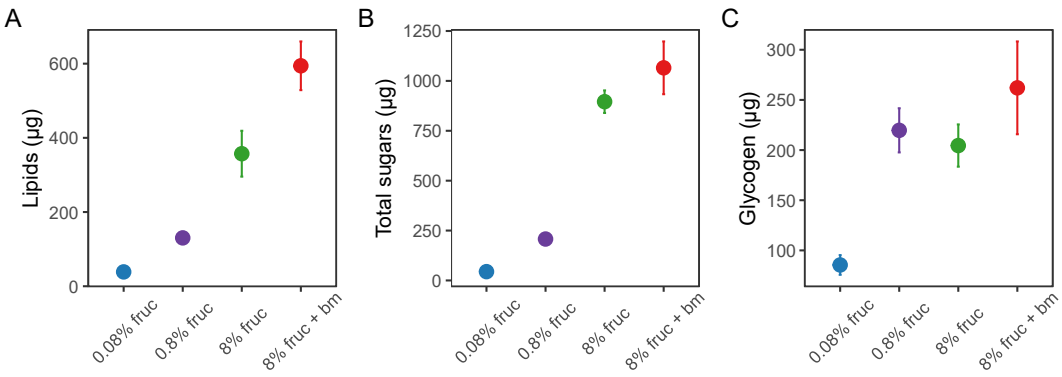

Figure 4.2. The body-size adjusted concentration (µg) per mosquito of lipids (A), total free sugars (B) and glycogen (C) in individually assayed mosquitoes under differing feeding treatments at day 9 post-uninfected blood meal (pBM). Data presented are mean ± SEM.

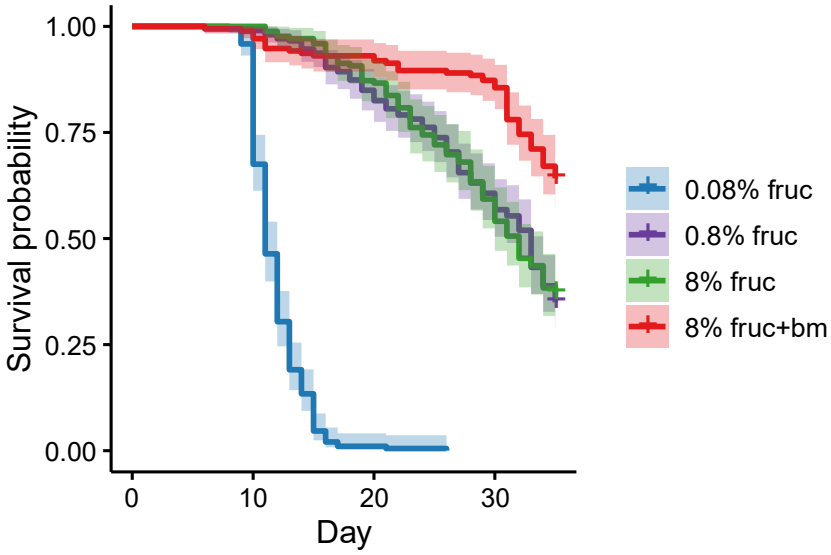

Figure 4.3. Mosquito survival in response to differing feeding treatments imposed following an initial meal of uninfected blood. Data presented are Kaplan-Meier curves ± 95% confidence intervals.

Table 4.1. Post hoc pairwise comparisons for nutritional content of mosquitoes provided with different feeding treatments. Significant p-values (<0.05) are highlighted in bold, and borderline p-values (0.05<p<0.09) are italicised and underlined.

		Test statistic	*p-value*
Lipids	0.08% fruc – 0.8% fruc	*t* = -3.25	**0.01**
	0.08% fruc – 8% fruc	*t* = -7.12	**<0.001**
	0.08% fruc – 8% fruc + bm	*t* = -10.8	**<0.001**
	0.8% fruc – 8% fruc	*t* = -3.82	**0.002**
	0.8% fruc – 8% fruc + bm	*t* = -7.41	**<0.001**
	8% fruc – 8% fruc + bm	*t* = -3.53	**0.005**
Total sugars	0.08% fruc – 0.8% fruc	*t* = -4.92	**<0.001**
	0.08% fruc – 8% fruc	*t* = -13.0	**<0.001**
	0.08% fruc – 8% fruc + bm	*t* = -14.3	**<0.001**
	0.8% fruc – 8% fruc	*t* = -7.98	**<0.001**
	0.8% fruc – 8% fruc + bm	*t* = -9.14	**<0.001**
	8% fruc – 8% fruc + bm	*t* = -1.04	0.73
Glycogen	0.08% fruc – 0.8% fruc	*t* = -4.42	**<0.001**
	0.08% fruc – 8% fruc	*t* = -4.04	**<0.001**
	0.08% fruc – 8% fruc + bm	*t* = -5.14	**<0.001**
	0.8% fruc – 8% fruc	*t* = 0.38	0.98
	0.8% fruc – 8% fruc + bm	*t* = -0.72	0.89
	8% fruc – 8% fruc + bm	*t* = -1.10	0.69

4.4.2 Resources affect parasite development within mosquitoes

First, we verified the assumption of our experimental design, that infection prevalence did not differ according to treatment group, ensuring that subsequent comparisons of parasite performance across treatment groups was not confounded by contributions of unequal proportions of infected mosquitoes. Overall oocyst prevalence was high, at 92±2%, and did not vary between treatments (χ^2_2=1.03, p = 0.60). While oocyst prevalence increased slightly between day 7 and 10 pIBM, from 89±3% to 96±1% (χ^2_1=5.79, p = 0.016), this did not differ across treatments (χ^2_2=0.36, p = 0.84) (Figure 4.4A) and is most likely due to oocysts becoming easier to identify as they grow.

Second, we examined parasite development in terms of oocyst burdens, growth rate, and coverage. We find that oocyst burden varied between feeding treatments in

manners that depended on day pIBM (χ^2_2=8.11, p = 0.017). Specifically, burden dropped between day 7 and 10 pIBM by 1.8-fold in mosquitoes on the low fructose diet (sqrt burden: 11.4±1.3 to 8.4±1.6), but only marginally (by 1.2-fold) in mosquitoes given an additional blood meal (sqrt burden: 12.9±1.3 to 11.6±1.3). In contrast, oocyst burden increased marginally (by 1.2-fold) in mosquitoes fed on 8% fructose (sqrt burden: 11.8±1.3 to 12.8±1.3) (Figure 4.4B, Table 4.2).

Oocyst diameter, which reflects growth rate, also differed across feeding treatments in manners that depended on day pIBM (χ^2_2=58.2, p < 0.001). Oocysts were larger on day 7 in mosquitoes provided with an additional blood meal (34.4±0.63μm) compared to those on fructose only diets (8% fruc: 31.2±0.62μm, low fruc: 30.4±0.65μm). From day 7 to day 10, oocysts in mosquitoes provided with an additional blood meal increased in size the least (by 6%). Oocysts in mosquitoes fed on 8% fructose grew at a similar rate to those provided an additional blood meal, increasing by 10% to reach a marginally smaller size on day 10 (8% fruc: 34.6±0.62μm, 8% fruc: + bm 36.5±0.62μm). In contrast, despite being small on day 7, oocysts grew at the fastest rate in the low fructose group, increasing by 16%, and reached a similar size to those in the additional blood meal group by day 10 (low fruc: 36.0±0.75μm) (Figure 4.4C, Table 4.2). Oocysts within a midgut had similar size variation regardless of feeding treatment (interaction: χ^2_2=1.44, p = 0.49, feeding treatment: χ^2_2=0.75, p = 0.69) and day pIBM (χ^2_1=1.13, p = 0.29) (Figure 4.4D).

Finally, we find that oocyst burden negatively correlated with oocyst size on day 7, where diameter was reduced by 0.006±0.003μm per additional oocyst (burden: χ^2_1=5.85, p = 0.016) equally across all treatments (χ^2_2=0.26, p = 0.88) (Figure 4.4E); a small difference in oocyst diameter is likely to be biologically significant because oocysts are spheres, causing small changes in diameter to result in large changes in volume. To account for this trade-off, we explored how patterns in total infection biomass, termed oocyst coverage (oocyst burden multiplied by mean oocyst volume per mosquito gut) differed over time. We find that oocyst coverage differed across feeding treatment in manners dependent on day pIBM (χ^2_2=7.57, p = 0.022). Coverage was approximately 1.8-fold higher in mosquitoes given an additional blood meal compared to those on a low fructose diet and stayed constant in these groups from day 7 to day 10. In contrast, oocyst coverage increased 1.4-fold in mosquitoes on 8% fructose diets, reaching a similar level to the coverage in mosquitoes provided with an additional blood meal by day 10 (sqrt oocyst coverage for 8% fruc: 2043±202μm^3, 8% fruc + bm: 2000±196μm^3) (Fig 4.4F, Table 4.2).

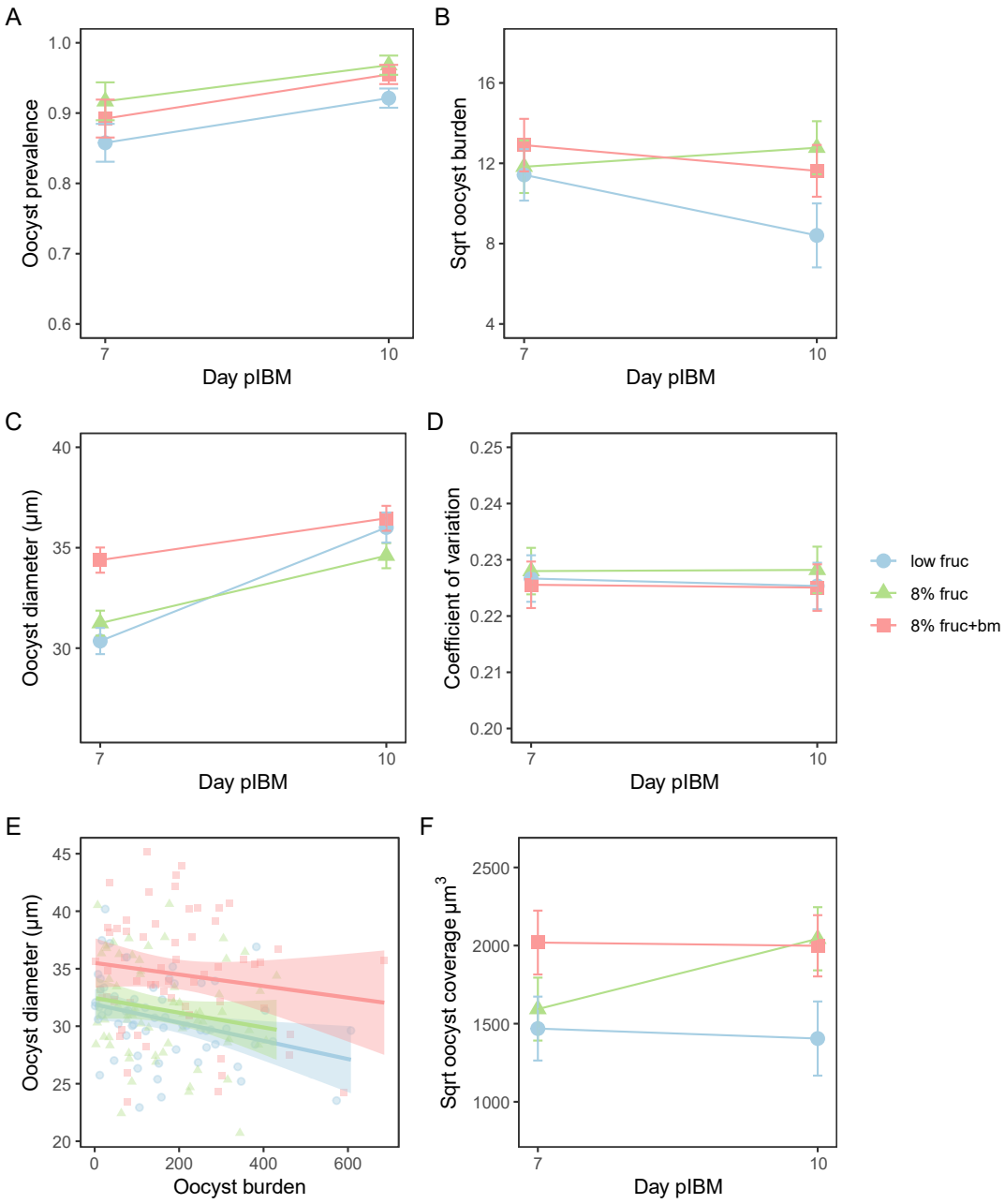

Figure 4.4. Oocyst development metrics from day 7 to day 10 post-infectious blood meal (pIBM) for mosquitoes subjected to different feeding treatments. Data presented are oocyst prevalence, defined as the proportion of infected mosquitoes (A), oocyst burden (B), oocyst diameter (C), and oocyst size variation as measured by the coefficient of variation (D), the correlation between oocyst burden and oocyst size on day 7 pIBM only (E) and oocyst coverage over time (F). To account for multiple measures per mosquito pot and per mosquito, data presented are predicted means ± SEM from the minimised models for (A), (B), (C), (D) and (F), and data points are minimised model predictions for individual mosquitoes, with lines and shading denoting best fit linear model estimates ± 95% confidence intervals for (E).

4.4.3 Resources shape onwards transmission potential

To test whether the developmental impacts of feeding treatments on parasites affects their onwards transmission potential, we compared the prevalence and the burden of sporozoites within mosquito salivary glands over time. Sporozoite prevalence was very high (98±5%) across all feeding treatments (interaction: χ^2_2=2.07, p = 0.36, feeding treatment: χ^2_2=3.18, p = 0.20), and stayed consistent over time (χ^2_1=0.16, p = 0.69) (Figure 4.5A). In contrast, sporozoite burden differed across feeding treatments in day pIBM-dependent manners (χ^2_2=9.61, p = 0.0082). Sporozoite burden was consistently higher in mosquitoes provided with an additional blood meal, despite a 1.6-fold reduction from day 11 to 14 pIBM (sqrt burden: 427±25 to 334±24) in this group. In contrast, for groups provided with only fructose, sporozoite burden stayed consistent from day 11 to 14 pIBM. By day 14 pIBM, compared to mosquitoes fed an additional blood meal, those provided with 8% fructose had approximately 1.7-fold fewer sporozoites (day 14 sqrt burden: 255±25), and sporozoite burden was 5.6-fold lower in mosquitoes fed on a low fructose diet (day 14 sqrt burden: 140±27) (Figure 4.5B, Table 4.2).

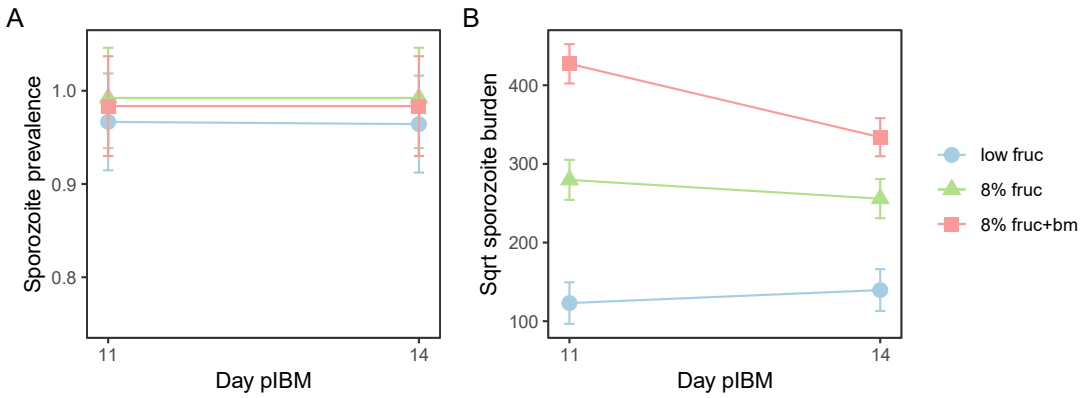

Figure 4.5. The impact of mosquito feeding treatments on sporozoite prevalence (A) and sporozoite burden (B) between day 11 and day 14 post-infectious blood meal (pIBM). Data presented are predicted means ± SEM from minimised models.

4.4.4 Linking parasite development and transmission potential

Finally, we explored whether the patterns we observe in the burdens and growth of oocysts can explain treatment group differences in sporozoite burden. We tested for correlations between oocyst metrics on day 10 and sporozoite burden on day 14, asking to what extent higher sporozoite burdens could be due to more productive oocysts in well-resourced mosquitoes. We find a positive correlation between oocyst and sporozoite burdens (χ^2_1=13.1, p<0.001), with the same slope across all feeding treatments (χ^2_2=3.40, p=0.18), and that oocyst productivity increased as access to resources increased (χ^2_2=16.8, p<0.001). Specifically, oocysts in mosquitoes provided an additional blood meal produced the most sporozoites, approximately 2.9-fold and 2-fold more than mosquitoes on the low fructose and 8% fructose diets, respectively (Fig 4.6A, Table 4.2). The relationships observed between oocyst and sporozoite burdens across feeding treatments are mirrored by oocyst coverage and sporozoite burden (oocyst coverage: χ^2_1=13.3, p<0.001; treatment: χ^2_2=15.6, p<0.001; oocyst coverage by treatment: χ^2_2=3.84, p=0.15) (Figure 4.6B, Table 4.2). In contrast, we find that oocyst size did not correlate with sporozoite burden (χ^2_1=0.009, p=0.93) within any feeding treatments (χ^2_2=3.80, p=0.15) (Figure 4.6C).

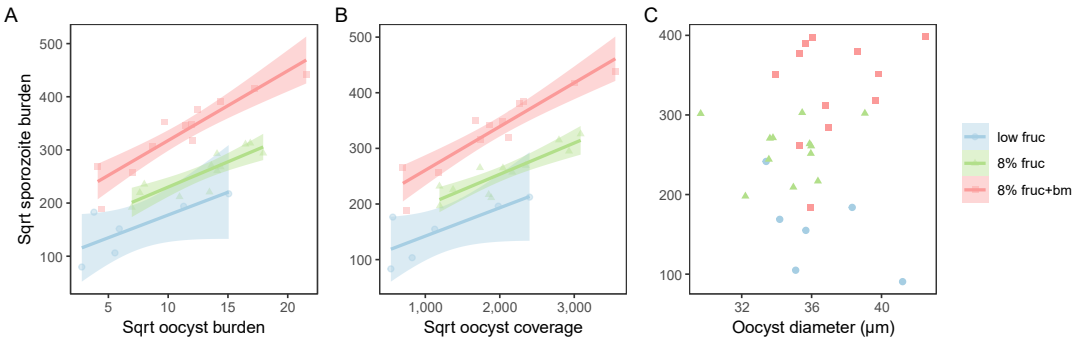

Figure 4.6. Correlations between sporozoite burden on day 14 pIBM and oocyst burden (A), overall oocyst coverage (B) and oocyst size (C) on day 10 pIBM for the differing mosquito feeding treatments. Data points are minimised model predictions per pot, taking into account multiple measures per pot of mosquitoes. Lines and shading denote best fit linear model estimates ± 95% confidence intervals.

Table 4.2. Post hoc pairwise comparisons for oocyst development and onward transmission potential across mosquito feeding treatments over time. Significant p-values (<0.05) are highlighted in bold, and borderline p-values (0.05<p<0.09) are italicised and underlined.

			Test statistic	*p-value*
Oocyst development	Oocyst burden	*Low fruc*		
		day 7 – day 10	*t* = 2.18	**0.03**
		8% fruc		
		day 7 – day 10	*t* = -1.44	0.15
		8% fruc + bm		
		day 7 – day 10	*t* = 1.48	0.14
	Oocyst size	*day 7*		
		Low fruc – 8% fruc	*z* = 2.18	0.50
		day 7		
		Low fruc – 8% fruc + bm	*z* = -4.51	**<0.001**
		day 7		
		8% fruc – 8% fruc + bm	*z* = -3.44	**0.002**
		day 10		
		Low fruc – 8% fruc	*z* = -1.24	0.43
		day 10		
		Low fruc – 8% fruc + bm	*z* = 0.04	0.99
		day 10		
		8% fruc – 8% fruc + bm	*z* = -1.31	0.39
	Oocyst coverage	*Low fruc*		
		day 7 – day 10	*t* = 0.06	0.95
		8% fruc		
		day 7 – day 10	*t* = -3.52	**<0.001**
		8% fruc + bm		
		day 7 – day 10	*t* = 0.005	0.99
Onward transmission potential	Sporozoite burden (over time)	*Low fruc*		
		day 11 – day 14	*t* = -0.41	0.68
		8% fruc		
		day 11 – day 14	*t* = 1.01	0.31
		8% fruc + bm		
		day 11 – day 14	*t* = 4.03	**<0.001**
	Sporozoite burden (day 14)	*day 14*		
		Low fruc – 8% fruc	*t* = 3.36	**0.005**
		day 14		
		Low fruc – 8% fruc + bm	*t* = -3.52	**<0.001**
		day 14		
		8% fruc – 8% fruc + bm	*t* = -2.14	*0.09*
Linking parasite development with onward transmission	Oocyst burden vs. sporozoite burden	Low fruc – 8% fruc	*t* = 1.06	0.55
		Low fruc – 8% fruc + bm	*t* = -3.66	**0.008**
		8% fruc – 8% fruc + bm	*t* = -3.22	**0.01**
	Oocyst coverage vs. sporozoite burden	Low fruc – 8% fruc	*t* = 1.27	0.43
		Low fruc – 8% fruc + bm	*t* = -3.61	**0.008**
		8% fruc – 8% fruc + bm	*t* = -2.97	**0.02**

4.5 Discussion

Here we examine how variation in resource availability within the mosquito affects components of vectorial capacity and the development of malaria parasites. Our feeding treatments perturbed mosquito nutritional status and survival. We show that resource availability influences mosquito survival in line with previous studies (Ebrahimi et al., 2018; Chisulumi et al., 2022; Yan et al., 2023); mosquitoes receiving the lowest fructose concentration contained less lipids and total sugars, and over 50% did not live long enough for sporogony to complete (10-14 days). While mosquitoes on intermediate fructose diets did live long enough for sporogony completion, mosquitoes receiving an additional blood meal had higher lipid stores than mosquitoes on fructose-only diets, and the longest lifespan (Figure 4.2,4.3). We also find that oocyst development follows different trajectories dependent on resource availability. Despite a trade-off between oocyst number and size in all treatments (Figure 4.4), parasites developing in mosquitoes with an additional blood meal had more productive oocysts; they reached a larger size earlier in development, and more sporozoites reached the salivary glands despite having a similar oocyst burden and final size to those in mosquitoes fed 8% fructose (Figure 4.4,4.5). When mosquitoes receive only sugar resources, oocyst development is affected in complex ways but the overall impact on sporozoite density correlates with the amount of sugar provided. In very poorly resourced mosquitoes, oocyst burden dropped during development, but oocysts grew more quickly between days 7-10 pIBM than those in mosquitoes on 8% fructose and in mosquitoes provided with an additional blood meal (Figure 4.4). The different responses of parasites in the best (i.e. consistent oocyst burden and large size by day 7) and the least (i.e. loss of oocysts coupled with catch-up growth) resourced mosquitoes resulted in total parasite biomass in the gut (oocyst coverage) remaining consistent over time for these groups, while coverage increased when mosquitoes had plentiful sugar without an additional blood meal (Figure 4.4). Oocyst productivity increased with resources; more sporozoites consistently reached the salivary glands in mosquitoes provided an additional blood meal (Figure 4.6). However, this was not explained by differences in oocyst size because both the best and least resourced mosquitoes had oocysts of the same size by the end of development (Figure 4.4).

In addition to the vector living long enough, transmission requires successful development of the parasite within the vector. Like previous studies using *P. falciparum*, we show that an additional blood meal increases *P. chabaudi* oocyst size earlier in sporogony (Shaw et al., 2020; Habtewold et al., 2021; Kwon et al., 2021).

This suggests parasites can utilise additional resources provided by a blood meal to develop more rapidly. Specifically, malaria parasites require lipids for generating membranes for subdivision into sporozoites (Shaw et al., 2020), so additional lipids from the blood meal may alleviate a resource constraint that prevents parasites from replicating within oocysts. Surprisingly, we also find that later in development, oocyst size increases most rapidly in mosquitoes on the lowest fructose diet. These parasites are likely to be resource limited and we suggest that the corresponding reduction in oocyst burden in this group alleviates resource constraints and allows surviving oocysts to rapidly reach maturity. The mechanism underpinning the reduction in oocyst numbers is unknown; some oocysts could starve and subsequently be degraded, and/or late-stage immune responses of mosquitoes could target developing oocysts (Gupta et al., 2009; Smith et al., 2015), both of which coincidently benefit surviving parasites. However, poorly-resourced mosquitoes are unlikely to be able to invest more in immune function than those given an additional blood meal because mosquitoes in a poor nutritional state have downregulated immune pathways (Yan et al., 2023), a reduced melanisation response (Koella and Sørensen, 2002), and increased susceptibility to pathogens likely via a reduction in immune investment (Muturi et al., 2011). Alternatively, malaria parasites have evolved sophisticated strategies during other life stages to optimise survival and transmission in the face of variable resource supply (Schneider and Reece, 2021), and previous studies suggest that parasites undergo programmed cell death at the ookinete stage to regulate infection intensity (Pollitt et al., 2010; Reece et al., 2011). While ‘suicidal’ apoptosis has not yet been observed in oocysts (Kakani et al., 2016), it may be beneficial to reduce oocyst number, trading off quantity for quality, to reduce competition for limited resources and ensure that the remaining oocysts are successful. Testing whether parasites in poorly-resourced mosquitoes display markers of cell death or are labelled by mosquito immune markers could disentangle whether the reduction in oocysts is due to a parasite adaptive response to resource limitation, killing by mosquito immune responses and/or oocysts simply starving and degrading due to lack of resources available (i.e. resource constraints). Further work should also consider the impact of resource availability across a range of starting oocyst densities, and how these interact to modulate development (Pollitt et al., 2013).

In addition to potentially modulating oocyst density, our finding that oocysts follow different size trajectories suggest that parasites may plastically alter oocyst growth rate in response to resource variation. For example, oocyst coverage changed (increased) over time only when mosquitoes received an intermediate resource

supply (8% fructose), remaining static in the other groups. Perhaps a lack of resources from blood is a constraint that forces oocysts to develop slowly, or they reach a size where they adaptively stop and wait for richer resources, only committing to complete development as best as possible if a blood meal is not acquired. Even though oocyst size and coverage in mosquitoes fed 8% fructose eventually caught up with those in mosquitoes receiving an additional blood meal, they produced fewer sporozoites, as also observed for *P. falciparum* (Kanatani et al., 2024) and *P. berghei* (Shiau et al., 2024). Our findings that lipid levels varied more across feeding treatments than total sugars and glycogen suggests lipids are an important resource during oocyst development. Restricted lipid trafficking in mosquitoes results in smaller, less productive oocysts which produce less infectious and less virulent sporozoites (Costa et al., 2018), and is consistent with the hypothesis that parasites are constrained, even when sugar is plentiful. However, other studies show that parasites become dormant under sugar-only conditions but can rapidly exploit an additional blood meal even later in development than in our study (Habtewold et al., 2021). A rapid response to a change in resource availability may also explain why oocysts in very poorly-resourced mosquitoes increased in size the fastest following a reduction in their number. Investigating the quality and quantity of sporozoites from mosquitoes under different feeding regimes would help elucidate whether parasites adjust their development in beneficial ways in response to resources or are simply directly constrained by resource availability.

Mosquitoes harbouring higher numbers of sporozoites in their salivary glands are more likely to infect a vertebrate host (Aleshnick et al., 2020; Kanatani et al., 2024), and high density infections may lead to faster within-vector development (Andolina et al., 2024). Therefore, resource availability may underpin a significant amount of heterogeneity in malaria transmission, through additive impacts on parasite development and mosquito behaviour/survival. Accounting for this variability in epidemiological models of vector-borne diseases may improve predictions of transmission and the impacts of vector control tools (Cator et al., 2020). For example, vector control tools which disrupt mosquito metabolism (e.g. insecticides (Ingham et al., 2021b)) or mosquito foraging (e.g. bed nets (Killeen et al., 2007)) may interrupt transmission by reducing resources available to parasites, and subsequently sporozoite burden.

Our resource perturbations also uncover that oocyst metrics can only partly explain patterns in sporozoite burden, highlighting that traditionally used parameters (such as oocyst burden) are not the best predictor of transmission potential. Instead, sporozoite

burden may be more appropriate, especially given that it correlates with infectivity to a vertebrate host (Aleshnick et al., 2020; Kanatani et al., 2024). Studies that test for trade-offs between oocyst metrics, the quantity of sporozoites and their quality (i.e. ability to reach the salivary glands), oocyst rupture success and/or the number of sporozoites per oocyst, are needed to understand what underpins how different developmental trajectories determine transmission potential. For example, lipid deprivation reduces sporozoite metabolic activity and infectivity to a vertebrate host (Costa et al., 2018), suggesting that parasites deprived of resources from multiple blood meals may produce fewer and less infective sporozoites. We also found that lipid stores differed across our feeding treatments, providing further support that lipid levels can drive differences in sporozoite burden. Furthermore, future studies should consider whether the failure of some oocysts in the gut to rupture (Andolina et al., 2024) could be a parasite strategy. For example, entering reversible dormancy during nutrient-limiting conditions (Habtewold et al., 2021) could help spread out sporozoite egress to reduce the immune shock to the vector, manage the demands made by sporozoites during migration to the salivary glands, and prevent crowding in the salivary glands. Performing sporozoite motility assays (e.g. (Beyer et al., 2021)), investigating patterns of oocyst rupture (Andolina et al., 2024) and the effects of mosquito immunity on sporozoites (Hillyer et al., 2007), and isolating oocysts (Siden-Kiamos et al., 2020) to investigate per oocyst productivity could begin to tease apart how resource availability influences transmission potential.

Overall, our study highlights that the best-resourced mosquitoes are likely to have a higher vectorial capacity than those with reduced access to resources, and that parasites can efficiently utilise additional resources available to them. Our results also suggest that parasites may adjust their development to suit their resource supply when facing limited conditions. While not directly investigated here, the nutritional status of mosquitoes can impact other components of vectorial capacity; for example, starved mosquitoes are more likely to host seek compared to well-resourced mosquitoes (Gary and Foster, 2001; Stone et al., 2012) (see Chapter 5). These effects are likely to synergise with the impacts of resources on parasite activities to generate complex consequences for transmission. Beyond malaria, many other vector-borne parasites (e.g. dengue virus, zika virus, *Leishmania* parasites) utilise resources from their vector (Serafim et al., 2018; Armstrong et al., 2020; Brackney et al., 2021), for replication/development (Herd et al., 2021). Vector-borne parasites/pathogens experience significant variation in resource availability within their vectors (due to a variety of factors, including age (Carrillo-Bustamante et al., 2023), climate (Barr et al.,

2023) and insecticide resistance mechanisms (Rivero et al., 2011; Tchouakui et al., 2020; Ingham et al., 2021b)). Thus, understanding the extent to which parasites are constrained and/or can adaptively adjust their development in response to vector condition is crucial for predicting disease transmission patterns, and parasites' evolutionary potential in the face of changes to their vectors (Oke et al., 2022).

Chapter 5. Propensity to host seek and biting time of day are modulated by *Anopheles gambiae* nutritional status

This chapter was the product of a collaboration between Yaw Afrane (University of Ghana Medical School), Samuel Rund (University of Notre Dame) and Maxwell Machani (Kenya Medical Research Institute), and was part-funded by a Varley-Gradwell Travelling Fellowship in Insect Ecology to Catherine Oke.

5.1 Abstract

Vector-borne disease transmission follows daily rhythms, with parasites/pathogens being taken up from an infected host at the time of day that vectors forage for blood meals, and passed to a new host during sequential, rhythmic, blood-feeding events. Vector control tools exploit rhythmic vector behaviour to reduce transmission, with insecticide-treated bed nets interrupting the nocturnal biting behaviour of *Anopheles* mosquitoes to significantly reduce transmission. Unfortunately, residual transmission still occurs, in part driven by mosquitoes shifting their biting to times of day when people are unprotected by bed nets. However, the extent to which mosquito temporal biology can shift is poorly understood. Daily foraging rhythms are a key determinant of fitness across many taxa and the time of day that foraging occurs is sensitive to physiological condition and resource availability. For mosquitoes, the availability of blood and sugar meals determine their lifespan and reproductive output, so we tested whether nutritional resources influence the time of day that host seeking behaviour occurs. We varied the amount of blood and sucrose that female *Anopheles gambiae s.l.* received and used baited traps in a semi-field system to quantify the time of day they seek a human host. We find that feeding treatments influenced both the likelihood and time of day that host seeking occurs. Specifically, low-resourced mosquitoes were 2-fold more likely to host seek than mosquitoes provided with high levels of sucrose and 3-fold more likely than those receiving high sucrose and an additional blood meal. Moreover, low-resourced mosquitoes were 5-10 fold more likely to host seek at an earlier time of day than well-resourced mosquitoes, which were predominantly host seeking in the second half of the night time. We supplemented our experimental data with preliminary field collections, confirming that the nutritional condition of wild-caught adult mosquitoes corresponds to that of experimentally manipulated mosquitoes fed 0.5% sucrose, and suggests that those with higher lipid levels host seek during the middle of the night (i.e. the ‘classical’ time window).

Overall, our results indicate that mosquito nutritional condition is an underappreciated contributor to residual malaria transmission by driving plasticity in biting time of day.

5.2 Introduction

The daily rotation of the Earth generates predictable rhythms in both abiotic factors (e.g. light, temperature, humidity) and biotic interactions (e.g. predation, mate availability, host-parasite-vector interactions), with organisms evolving circadian clocks to align their physiologies and behaviours with environmental periodicity (Vaze and Sharma, 2013). The role of daily rhythms in infections has been gaining traction, because rhythms dictate when hosts, parasites and vectors interact, and all parties exhibit rhythms in biological processes that can influence disease severity and transmission (Rund et al., 2016; Westwood et al., 2019; Prior et al., 2020). The transmission of vector-borne diseases (VBDs), such as malaria and dengue, is reliant on the foraging rhythms of insect vectors, which take up parasites/pathogens from an infected vertebrate host during a blood meal and transmit them to a new host during a subsequent blood-feeding event. For example, *Plasmodium* parasites, the causative agent of malaria, are vectored by members of the *Anopheles* genus, which are generally nocturnally active and preferentially bite between the hours of 11pm and 4am (i.e. their 'classical' time window) (Moiroux et al., 2012; Thomsen et al., 2017; Sangbakembi-ngounou et al., 2022). Control programmes, such as insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS), exploit these foraging and subsequent resting behaviours by bringing mosquitoes into contact with a lethal insecticide. ITNs and IRS are some of the most effective methods to curb the spread of malaria (Oke et al., 2022; World Health Organisation, 2023), averting 75% of deaths since 2000 (Bhatt et al., 2015). Despite this success, residual transmission still occurs, in part due to the evolution of genetic insecticide resistance and behavioural resistance in vector populations (Huijben and Paaijmans, 2018). The scale of residual transmission remains unclear, but heterogenous biting behaviour across mosquito populations may cause the efficacy of malaria control to vary (Sherrard-Smith et al., 2019).

There are increasing reports suggesting that *Anopheles* spp. are shifting their biting behaviour to earlier in the evening ('early' biting) or later in the morning ('late' biting) when humans are unprotected by bed nets (Moiroux et al., 2012; Yohannes and Boelee, 2012; Russell et al., 2013; Sougoufara et al., 2014; Cooke et al., 2015; Thomsen et al., 2017; Carrasco et al., 2019). While shifts in biting time of day may

promote transmission simply due to increased access to hosts, vector foraging at non-classical times of day affects multiple parameters of parasite fitness and transmission (often expressed as the basic reproductive number, R_0 (Smith et al., 2012)) in complex and potentially opposing ways (Rund et al., 2016). For example, mosquitoes are less susceptible to malaria infection at night (Schneider et al., 2018b), likely due to rhythmicity in immune defences (Murdock et al., 2013), but parasites are more infective to mosquitoes at night (Pigeault et al., 2018; Schneider et al., 2018b). Furthermore, disruption to foraging rhythms is detrimental to health and fitness across diverse taxa, including insects (Gill et al., 2015), and parasites rely on nutrients scavenged from their vector to proliferate and for onward transmission opportunities (Shaw et al., 2022) (see Chapter 4). Thus, if acquiring blood at a non-classical time of day detrimentally impacts the efficacy of blood digestion and mosquito health, vectors may not provide sufficient food resources for parasite proliferation nor live long enough for the completion of parasite development (termed 'sporogony'). Therefore, shifts in biting time of day could have significant impacts on vectorial capacity and the success of vector control tools.

While shifts in biting rhythms have a heritable element, they are predominantly driven by non-genetic factors (Govella et al., 2023), suggesting that mosquitoes use phenotypic plasticity to adjust their foraging behaviour in response to environmental conditions (Pigliucci, 2005), such as limited access to blood meals due to ITN use. However, the extent to which mosquito biting time of day can shift and the potential drivers are poorly understood, though data from other taxa suggest that physiological condition and resource availability can alter foraging rhythms (Van Der Veen et al., 2017). Altering the timing of their foraging can be adaptive (i.e. fitness enhancing), especially in resource-limiting conditions (Van Der Vinne et al., 2014). For example, low food availability promotes a shift to diurnal foraging patterns in small nocturnal rodents, which minimises energy loss by remaining in burrows during particularly cold nights (Van Der Vinne et al., 2015, 2019). Similarly, mosquitoes may garner greater benefits from shifting biting time of day to evade ITNs (or suffer fewer costs from disrupting their foraging rhythm) when they are in poor nutritional condition. Such context-dependent variation in the costs and benefits of shifts in biting time may explain why biting times have not significantly changed in some populations despite high ITN use (Sougoufara et al., 2016; Kreppel et al., 2020), and why the evolution of behavioural resistance varies across populations based on the abundance of mosquito nutrition sources (Stone et al., 2016).

The rhythmic feeding ecology of *Anopheline* mosquitoes (Yee and Foster, 1992; Gary and Foster, 2006; Yohannes and Boelee, 2012) is under circadian clock control (Rund et al., 2013) and revolves around the need for blood and sugar meals. Female mosquitoes utilise proteins and lipids in blood for egg production, and sugar sources power flight and the accumulation of energy reserves (Barredo and Degennaro, 2020). Females take a blood meal after mating, beginning their first gonotrophic cycle of blood feeding, egg development and oviposition, seeking more blood approximately every 3 days (Mitchell and Catteruccia, 2017). Malaria parasites rely on mosquito blood-feeding for transmission (Shaw et al., 2022); they are taken up during a blood meal and are ready to transmit to a new host after approximately 10-14 days, during a subsequent blood meal. As ITN use prevents mosquitoes from being obtaining a blood meal at their preferred time of day (Finda et al., 2019; Monroe et al., 2020; Murray et al., 2020; Okumu, 2020), blood meal availability is reduced (Killeen et al., 2007), limiting the resources available to mosquitoes and developing parasites. The type and abundance of nutritional resources available to mosquitoes also varies across habitats and seasons (Gu et al., 2011; Barredo and Degennaro, 2020). In response, mosquitoes exhibit plasticity in multiple feeding behaviours in ways which are adaptive; for example, failure to acquire a blood meal leads to increased sugar feeding (Gary and Foster, 2006) to promote survival until future biting opportunities. However, despite the importance of mosquito foraging rhythms for malaria transmission and evidence that physiological condition can alter foraging rhythms in other taxa, the effect of resource availability on mosquito biting time of day is unknown.

By perturbing the nutritional resources provided to mosquitoes, we investigate whether mosquito biting behaviour exhibits phenotypic plasticity in the time of day that host seeking occurs. We provided mosquitoes with a blood meal and then restricted access to subsequent blood meals and/or sugar, simulating scenarios in which mosquitoes have: (i) failed at multiple blood feeding attempts, (ii) failed at multiple blood feeding attempts but have access to a sugar-rich environment, and (iii) successfully acquired blood when their gonotrophic cycle requires it and have access to sugar. We reveal that mosquitoes with access to additional blood meals and/or high levels of sugar host seek during the classical biting window and into the morning, but when sugar and blood are limited, mosquitoes are more likely to host seek in the evening when humans are unlikely to be protected by ITNs. We also supplement our data with preliminary field collections that suggest biting time of day of wild-caught adult mosquitoes correlates with some aspects of nutritional condition. We discuss

the potential outcomes of shifts in biting time of day in response to resource availability for parasite fitness and transmission, the efficacy of ITNs, understanding residual transmission patterns, and for future-proofing vector control tools.

5.3 Methods

We performed semi-field assays and field collections of adult mosquitoes to assess if mosquito nutritional status impacts host seeking and biting time of day. First, we allocated female *Anopheles gambiae s.l.* mosquitoes to three resource treatments (0.5% sucrose, 10% sucrose or 10% sucrose plus an additional blood meal) and released them into an enclosed semi-field system with traps mimicking human odour. The traps were programmed to turn on at 6pm-10pm (evening), 11pm-3am (classical) and 4am-8am (morning) respectively, to catch mosquitoes intending to bite at different times across the night. Second, we performed a preliminary collection of wild adult *Anopheles* mosquitoes over the course of one night, to assess whether there was evidence of correlations between mosquito nutritional status and the time of day they attempted to bite.

5.3.1 Mosquitoes

Mosquitoes used in the host seeking assay were F2 progeny of wild-caught *Anopheles gambiae s.l.* larvae, collected from three sites across the Greater Accra region of Ghana (Tuba, 5° 30' 47" N 0° 23' 16" W; Teshie, 5° 35′ 0″ N, 0° 6′ 0″ W; East Legon, 5° 38' 16.39" N, 0° 9' 40.33" W) (Figure 5.1) in Dec 2023-Jan 2024. We maintained all generations of mosquitoes under standard rearing conditions (26±2°C, 80% relative humidity with a 12L:12D cycle), and blood meals were provided by direct human feeds, where a trained volunteer places their arm into or on top of mosquito cages (Harrington et al., 2020). We gave both F0 and F1 generations *ad libitum* 10% sucrose solution in water, and provided them with soaked cotton wool in petri dishes for egg laying. On day 1 post-emergence of the F2 generation, we transferred female mosquitoes to three experimental cages (n=100-110 per cage) and allocated each cage to one of three resource treatments: (i) 0.5% sucrose in water (0.5% suc), (ii) 10% sucrose in water (10% suc) or (iii) 10% sucrose in water plus an additional blood meal on day 6 post-emergence (10% suc + bm). We maintained all treatment groups on their feeding treatments from day 1 post-emergence until the host seeking assay, provided them all with a blood meal on day 3 post-emergence and gave them the opportunity to lay eggs. On day 9-10 post-emergence, we transferred mosquitoes

from each resource group to paper cups (n=70-100 per cup) and provided them with water only for six hours prior to release in the semi-field system. We conducted a total of six releases across six separate blocks of mosquitoes.

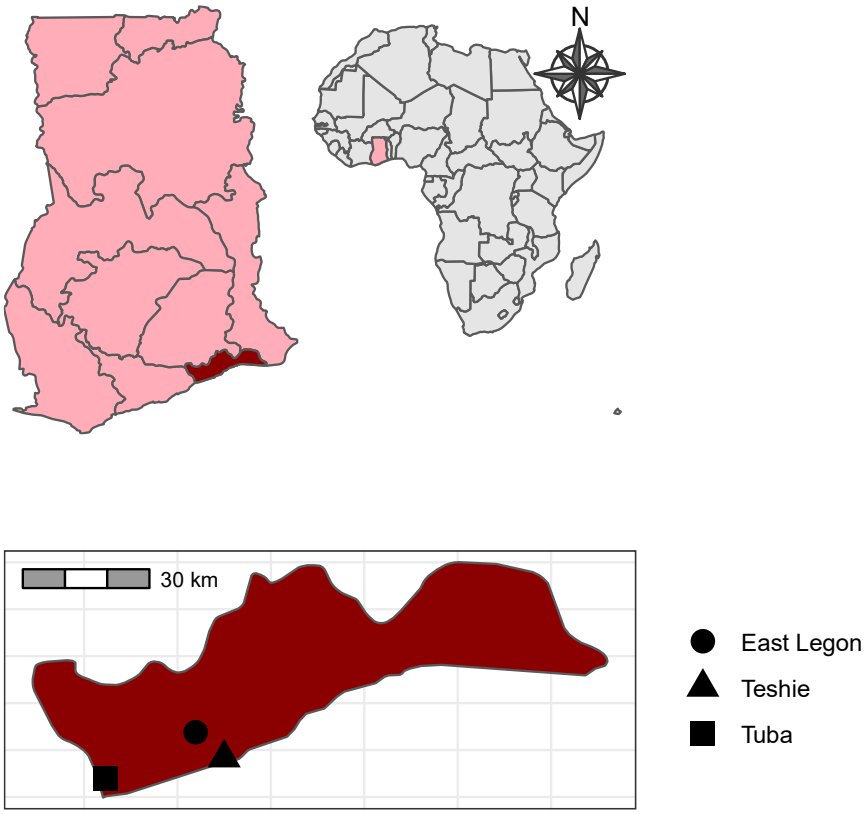

Figure 5.1. Map of larval and adult mosquito collection sites in Greater Accra, Ghana. Larvae from the three sites were collected in Dec 2023-Jan 2024, combined, and female F2 progeny were used for mosquito nutrition perturbations and behavioural assays. Wild adult mosquitoes were collected from Teshie across one night. Maps were created using the R packages *sf* (Pebesma, 2018), *ggspatial* (Dunnington, 2023) and *rnaturalearth* (Massicotte and South, 2023).

To investigate nutritional content in wild mosquitoes, we collected adult mosquitoes from one site in the Greater Accra region of Ghana (Teshie, 5° 35′ 0″ N, 0° 6′ 0″ W) (Figure 5.1) overnight between 6pm and 7am in February 2024, using the human landing catch (HLC) technique. This technique involves trained human collectors exposing their lower legs and collecting mosquitoes that land on them before they bite, and is the gold standard for investigating human exposure to disease vectors (Gimnig et al., 2013). Every hour, we snap-froze collected mosquitoes on dry ice and

identified specimens to species level according to morphological characteristics (Gillies and Coetzee, 1987), noting the number of *Anopheles* spp. mosquitoes collected per hourly bin. We placed individual *Anopheline* mosquitoes in 1.5ml microcentrifuge tubes and stored them on dry ice, transferring them to a -20°C freezer after the overnight collection period had ended.

5.3.2 Semi-field host seeking assay

We conducted mosquito behavioural experiments in January-February 2024 at the University of Ghana in an enclosed semi-field system dubbed the MalariaSphere (Machani et al., 2022; Osoro et al., 2022). The average maximum and minimum daily temperatures during the semi-field releases were 34.5±0.4°C and 25.8±0.6°C respectively (Valer, 2024). The enclosure of 5.8 x 4.2 x 2.8m was covered in an insect-proof screen to prevent mosquito escape and/or entry from the external environment. We placed three BG-Sentinel traps (Biogents, Germany), with a MB5 lure (Biogents, Germany) and a CO_2 flow of approximately 500g/day to mimic human scent and breath, in the enclosure with different traps programmed to be on during each of (i) 6pm-10pm, (ii) 11pm-3am, or (iii) 4am-8am, to capture mosquitoes biting in the evening ('early' biters), the 'classical' biting time window during the night, and morning ('late' biters) respectively (Figure 5.2). CO_2 flow was controlled with BG-CO_2 timers (Biogents, Germany), ensuring that gas was only flowing when its respective trap was switched on. To provide mosquitoes with potential resting sites, we added four clay pots to the enclosure. We did not provide water or food sources in the enclosure, because this would have confounded the experimental treatment groups. We colour-marked mosquitoes using fluorescent powder (FTX series, Swada London), allowing us to distinguish between the different treatment groups, and applied the powder following Machani *et al* (Machani et al., 2022), rotating the colours across sequential releases to ensure that powder colour did not confound the impacts of each experimental treatment on mosquito behaviour. We carried out six releases over a period of three weeks, with mosquitoes being released into the enclosure between 5pm-5:30pm (n=235-300 mosquitoes per release). A period of at least 48 hours between each release and checking all clay pots with a Prokopack aspirator ensured that any remaining untrapped mosquitoes had been removed or died before the next release. We collected mosquitoes from traps at 8am the day after the release, then cold-anaesthetised and counted them.

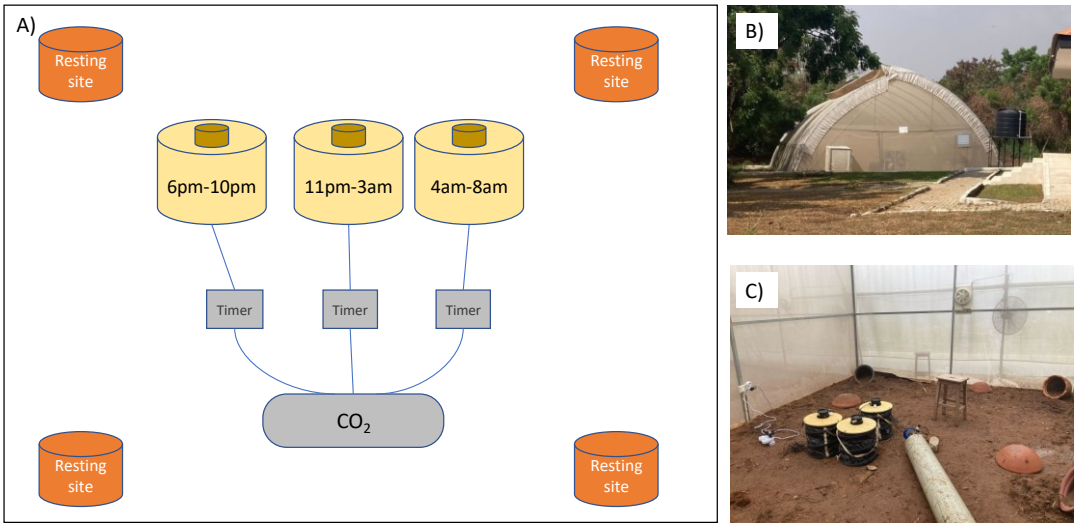

Figure 5.2. The semi-field behavioural assay set-up. Schematic diagram of the set-up (A), featuring four resting sites for mosquitoes, and traps baited with human odour which were programmed to turn on at 6pm-10pm, 11pm-3am and 4am-8pm respectively. All traps were linked to a CO_2 source, which was split to three timers ensuring the CO_2 was only flowing when the trap that was switched on. Photographs show the semi-field facility (B) and the behavioural assay set-up (C).

5.3.3 Nutrition assays

We quantified mosquito nutritional status in a subset of mosquitoes from each feeding treatment group (n=14-19 per group, total n=48) prior to semi-field releases to confirm our resource perturbations impacted nutritional status, and from wild-caught mosquitoes (n=66), to assess if nutritional content differed across mosquitoes biting at different times of day. All mosquitoes were frozen at -20°C for lipid, glycogen and total sugar analyses following modified Van Handel protocols (Van Handel, 1985a, 1985b; Van Handel and Day, 1988). In brief, we removed a wing from each mosquito to quantify body size, and lysed individual mosquitoes in 100µl 2% sodium sulphate and added 750µl of 1:2 chloroform:methanol. We centrifuged samples at 12000 rpm for 3 minutes; the supernatant was used for lipid and total sugars analysis, and the precipitate was used for glycogen analysis. We conducted lipid analysis following

Meuti *et al* (Meuti et al., 2015) with minor modifications: we used 250µl supernatant, and added 80µl of sample to 96-well plates before measuring absorbance at 525nm and 490nm on a microplate reader (Varioskan Lux, ThermoFisher Scientific). We carried out glycogen analysis by adding 200µl anthrone reagent to the precipitate, and heating to 90°C for 15 minutes. After cooling at 4°C for 5 minutes, we added 40µl to a 96-well plate containing 40µl anthrone reagent and measured absorbance at 625nm and 555nm on a microplate reader. Total sugars analysis followed a similar protocol to glycogen with minor differences: we heated 250µl (wild-caught mosquitoes) or 200µl (semi-field mosquitoes) of supernatant at 90°C until the solvent evaporated, and then added 200µl anthrone reagent. After heating and cooling as above, we added 80µl to 96-well plates before measuring absorbance at the same wavelengths.

5.3.4 Statistical analysis

We used R v. 4.1.3 to perform data analysis. We analysed nutritional content (lipid, glycogen, total sugars) of individual mosquitoes corrected for body size (approximated by wing length) using linear mixed models with feeding treatment as a main effect and mosquito release batch as a random effect for mosquitoes from the host seeking assay, and linear models with biting time of day as an unordered factor for our wild mosquito collection data. We conducted preliminary analysis to see how nutritional content between field-caught and lab-reared mosquitoes varied, using linear models to compare between the field-caught, 0.5% suc, 10% suc and 10% suc + bm groups. We performed a chi-square test to investigate if the number of field-caught mosquitoes differed throughout the night. Specifically, we grouped hourly bins into the evening (6pm-11pm), classical (11pm-4am) and morning (4am-7am) time windows.

For the host seeking assay, we used binomial generalised linear mixed models (glmm) with mosquito release batch as a random effect to investigate how feeding treatment impacted the overall proportion of mosquitoes caught across the night, with feeding treatment as a main effect, and how feeding treatment impacted the proportion of mosquitoes caught across the different biting time of day windows, with feeding treatment, trap time and their interaction as main effects. Proportion of caught mosquitoes and relative odds ratios (OR) ± SE were estimated from models using the *emmeans* (Lenth, 2023) package.

Nutrition data were square root transformed to meet assumptions of normality and homogeneity of variance. We minimised all models using likelihood ratio tests, and

AICc for non-nested models, and tested for conformation to model assumptions using the *easystats* (Makowski et al., 2020) package. To account for differences between mosquito release batches in the semi-field experiment, we present estimated marginal means ± SEM (*emmeans* package), predicted from models. We conducted post hoc pairwise comparisons using the Tukey method with the *emmeans* package whenever the minimised model contained a significant effect, and to compare nutritional content between field-caught and lab-reared mosquitoes.

5.3.5 Ethics statement

The study protocols for HLCs and mosquito colony blood feeds were ethically reviewed and approved by the Ghana Health Service Ethics Review Committee (GHS-ERC: 021/07/23), and by the University of Edinburgh Ethics Committee (sreece-0003). All volunteers/collectors were consenting, trained research staff at the University of Ghana.

5.4 Results

5.4.1 Resource availability perturbs mosquito nutritional status

We investigated how our resource perturbations affected mosquito physiological condition. Mosquitoes fed on the lowest concentration of sucrose (0.5%) had significantly lower lipid levels (55.5±17.8µg) (χ^2_2=17.6, p<0.001), approximately 2.5-fold lower than those fed with 10% sucrose (138±31.4µg) and 3.3-fold lower those fed an additional blood meal (182±29.6µg) (Figure 5.3A, Table 5.1). A similar pattern was observed for total sugars: mosquitoes fed with 0.5% sucrose had the lowest total sugar levels (85.6±69.9µg) (χ^2_2=30.8, p<0.001), approximately 5-fold lower than those given 10% sucrose (420±158µg) and an additional blood meal (439±156µg) (Figure 5.3B, Table 5.1). Finally, mosquitoes fed with 0.5% sucrose had the lowest glycogen levels (105±64.2µg) (χ^2_2=21.7, p<0.001), approximately 7-fold lower than those given 10% sucrose (754±187µg), with mosquitoes given an additional blood meal exhibiting intermediate levels (481±128µg) (Figure 5.3C, Table 5.1).

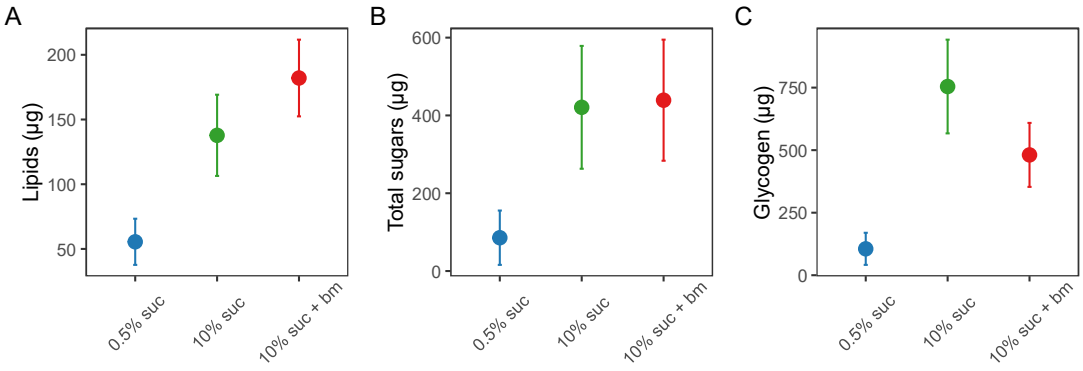

Figure 5.3. The body size adjusted concentrations (µg) per mosquito of lipids (A), total free sugars (B) and glycogen (C) in individual mosquitoes under differing feeding treatments on day 10 post-emergence. Nutritional perturbations began on day 1 post-emergence and all treatment groups received a blood meal on day 3 post-emergence (with an additional blood meal given to the 10% suc + bm group on day 6). Data presented are estimated marginal means ± SEM.

Table 5.1. Post hoc pairwise comparisons for nutritional content of mosquitoes provided with different feeding treatments. Significant p-values (<0.05) are highlighted in bold, and borderline p-values (0.05<p<0.09) are italicised and underlined.

		Test statistic	***p-value***
Lipids	0.5% suc – 10% suc	*t* = 2.75	**0.02**
	0.5% suc – 10% suc + bm	*t* = -4.54	**<0.001**
	10% suc – 10% suc + bm	*t* = -1.16	0.48
Total sugars	0.5% suc – 10% suc	*t* = 5.13	**<0.001**
	0.5% suc – 10% suc + bm	*t* = -6.13	**<0.001**
	10% suc – 10% suc + bm	*t* = -0.21	0.98
Glycogen	0.5% suc – 10% suc	*t* = 4.66	**<0.001**
	0.5% suc – 10% suc + bm	*t* = -3.69	**0.002**
	10% suc – 10% suc + bm	*t* = 1.55	0.28

5.4.2 *Host seeking and biting time of day are impacted by mosquito resource availability*

We tested how mosquito nutritional status impacted the tendency and time of day that mosquitoes seek a blood meal using the proportion of trapped mosquitoes from each feeding treatment as a proxy for successfully locating a host. Across the six releases and all traps, 32.2% of mosquitoes (506/1569) were caught. Across all releases and times of day, we found the likelihood to host seek correlated negatively with the level of nutritional resources provided (χ^2_2=65.8, p<0.001) (Figure 5.4A). Specifically, mosquitoes fed 0.5% sucrose were 2-fold more likely to be trapped than those fed 10% sucrose (odds ratio (OR): 2.08±0.28) and 3-fold more likely than those fed an additional blood meal (OR: 2.97±0.42), and mosquitoes fed 10% sucrose were 1.4-fold more likely to be trapped than those fed an additional blood meal (OR: 1.43±0.21) (Table 5.2).

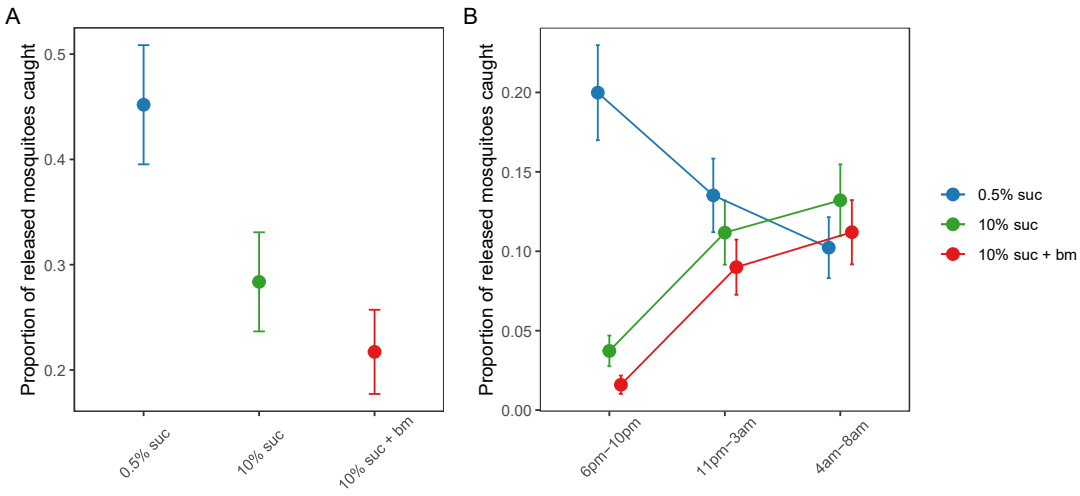

Figure 5.4. The proportion of released mosquitoes from each feeding treatment that were trapped across the entire night (A) and that were caught in the evening (6pm-10pm), classical night time biting window (11pm-3am) and morning (4am-8am) (B). Data presented are estimated marginal means ± SEM.

We also found the proportion of mosquitoes caught at different times-of-day varied across feeding treatments (χ^2_4=95.4, p<0.001) (Figure 5.4B). Specifically, mosquitoes fed 0.5% sucrose were approximately 5- and 10-fold more likely to be caught in the evening (20±2.9%) than those fed with 10% sucrose (3.7±0.9%) and an additional

blood meal (1.6±0.5%), respectively (Table 5.2). Mosquitoes fed 10% sucrose with or without an additional blood meal followed similar temporal patterns, with approximately 4-fold more of these mosquitoes being trapped during the classical biting window (10% sucrose: 11±2.0%, 10% sucrose + bm: 9.0±1.7%) and in the morning (10% sucrose: 13±2.3%, 10% sucrose + bm: 11±2.0%) than in the evening (10% sucrose: 3.7±0.9%, 10% sucrose + bm: 1.6±0.5%) (Table 5.2). In contrast, most mosquitoes fed 0.5% sucrose were trapped in the evening (20±2.9%), compared to during the classical biting window (13±2.3%) and in the morning (10±1.9%) (Table 5.2).

Table 5.2. Post hoc pairwise comparisons between feeding treatments for propensity to host seek and biting time of day. Significant p-values (<0.05) are highlighted in bold, and borderline p-values (0.05<p<0.09) are italicised and underlined.

			Test statistic	***p-value***
Propensity to host seek		0.5% suc – 10% suc	*z* = -5.42	**<0.001**
		0.5% suc – 10% suc + bm	*z* = 7.72	**<0.001**
		10% suc – 10% suc + bm	*z* = 2.46	**0.04**
Biting time of day (differences across trapping times)	6pm-10am	0.5% suc – 10% suc	*z* = -7.48	**<0.001**
		0.5% suc – 10% suc + bm	*z* = 7.72	**<0.001**
		10% suc – 10% suc + bm	*z* = 2.16	*0.08*
	11pm-3am	0.5% suc – 10% suc	*z* = -1.16	0.48
		0.5% suc – 10% suc + bm	*z* = 2.32	*0.05*
		10% suc – 10% suc + bm	*z* = 1.19	0.46
	4am-8am	0.5% suc – 10% suc	*z* = 1.50	0.29
		0.5% suc – 10% suc + bm	*z* = -0.51	0.86
		10% suc – 10% suc + bm	*z* = 1.01	0.57
Biting time of day (differences across feeding treatments)	0.5% sucrose	6pm-10pm – 11pm-3am	*z* = -2.76	**0.02**
		6pm-10pm – 4am-8am	*z* = 4.31	**<0.001**
		11pm-3am – 4am-8am	*z* = 1.63	0.23
	10% sucrose	6pm-10pm – 11pm-3am	*z* = -4.52	**<0.001**
		6pm-10pm – 4am-8am	*z* = -5.34	**<0.001**
		11pm-3am – 4am-8am	*z* = -1.02	0.56
	10% sucrose + bm	6pm-10pm – 11pm-3am	*z* = 4.92	**<0.001**
		6pm-10pm – 4am-8am	*z* = -5.66	**<0.001**
		11pm-3am – 4am-8am	*z* = -1.21	0.45

5.4.3 Biting time of day distribution and nutritional status of wild-caught Anopheles spp.

We caught a total of 94 wild mosquitoes during our overnight collection. As expected, the majority of mosquitoes were caught biting during the classical window and peaked between 12am-1am (Figure 5.5). We grouped hourly bins into the evening (6pm-11pm), classical (11pm-4am) and morning (4am-7am) time windows and found that there were differences in the number of mosquitoes caught across these windows (χ^2_2=45.0, p<0.001). Specifically, 68% of mosquitoes were caught during the classical biting window, compared to 7% and 24% caught during the evening and morning windows respectively.

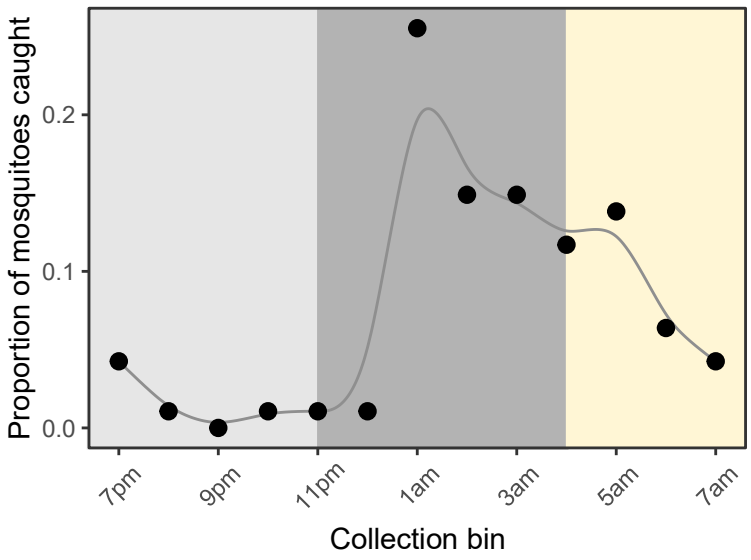

Figure 5.5. The proportion of wild adult *Anopheles spp*. mosquitoes caught across hourly bins between 6pm and 7am. To highlight how the pattern changes over the course of the night, data points are connected by an X-spline. Evening, classical, and morning bins are indicated by shaded regions from left to right.

Our preliminary data suggest that mosquitoes vary in nutritional content, but there were no differences between lipid ($F_{9,56}$=0.75, p=0.66), sugars ($F_{9,56}$=1.06, p=0.40) or glycogen ($F_{9.56}$=0.40, p=0.93) contents across the hourly bins (Figure 5.6). However, there is a suggestive trend for mosquitoes biting in the evening or morning to have approximately 1.2-fold lower lipid levels compared to those biting in the classical window (Figure 5.6A). We also found that the nutrient contents of mosquitoes in the field was similar to our experimental, lab-reared, mosquitoes fed on 0.5% sucrose

(estimated marginal means for field-caught mosquito lipids: 39.8±5.5µg, sugars: 65.4±13µg, and glycogen: 51.5±11µg), but was significantly lower than mosquitoes fed 10% sucrose and 10% sucrose plus a blood meal (lipid: $F_{3,110}$=24.8, p<0.001; sugars: $F_{3,110}$=25.9, p<0.001; glycogen: $F_{3,110}$=58.1, p<0.001; Table 5.3). Specifically, field-caught mosquitoes had approximately 4-fold lower lipid levels, 6.5-fold lower total sugars and 12-fold lower glycogen levels.

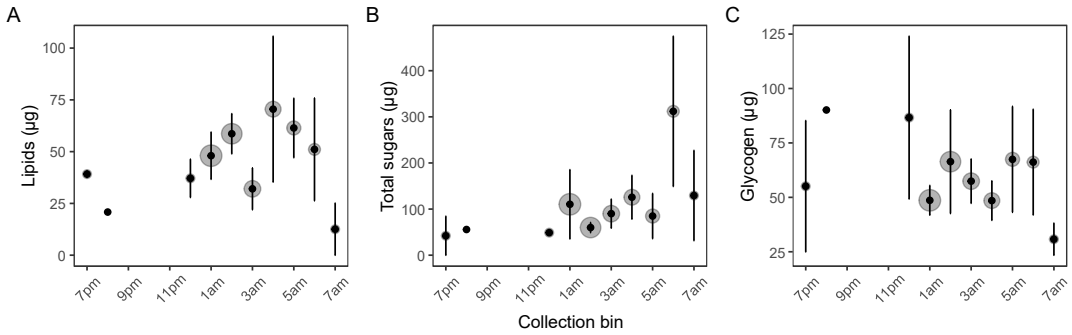

Figure 5.6. The body size adjusted concentrations (µg) per mosquito of lipids (A), total free sugars (B) and glycogen (C) in individual wild-caught adult *Anopheles spp.* mosquitoes. Data presented are means ± SEM. The size of the grey circle reflects the sample size per hourly bin.

Table 5.3. Pairwise comparisons of the nutritional content of field-caught mosquitoes versus. lab-reared mosquitoes on different feeding treatments. Significant p-values (<0.05) are highlighted in bold, and borderline p-values (0.05<p<0.09) are italicised and underlined.

		Test statistic	***p-value***
Lipids	field – 0.5% suc	*t* = -1.25	0.59
	field – 10% suc	*t* = -4.82	**<0.001**
	field – 10% suc + bm	*t* = -7.91	**<0.001**
Total sugars	field – 0.5% suc	*t* = -1.13	0.67
	field – 10% suc	*t* = -4.95	**<0.001**
	field – 10% suc + bm	*t* = -8.06	**<0.001**
Glycogen	field – 0.5% suc	*t* = -1.96	0.21
	field – 10% suc	*t* = -11.3	**<0.001**
	field – 10% suc + bm	*t* = -8.86	**<0.001**

5.5 Discussion

By altering sugar provision and access to blood meals, we tested how resource availability impacts the tendency and time of day that mosquitoes host seek. We find that mosquitoes fed on a low sucrose diet with no access to a second blood meal had the lowest levels of lipids, sugars and glycogen (Fig 5.3). Providing a higher concentration of sucrose had the greatest impact on nutritional reserves, increasing lipid, glycogen and sugar levels and an additional blood meal did not increase nutritional reserves further (Figure 5.3). As resources increased, propensity to host seek reduced; mosquitoes on a low sucrose diet were the most likely to be caught in traps mimicking human breath and scent, followed by mosquitoes given a higher concentration of sucrose, and then those with access to sucrose and an additional blood meal (Figure 5.4A). We also found that resource availability modulates the time of day that mosquitoes search for a blood meal. Mosquitoes on a low sucrose diet were more likely to host seek in the evening, whereas better-resourced mosquitoes were most likely to host seek during the classical biting time window and continue into the morning (Figure 5.4B). Finally, our preliminary collections of wild adult female *Anopheline* mosquitoes highlights that there is variation in mosquito biting time and nutritional status in a natural population in Ghana (Figure 5.5,5.6). There was a suggestive trend that mosquitoes biting outside of the classical time window have lower lipid levels (Figure 5.6A). Furthermore, we find that wild mosquitoes have similar nutrient levels to lab-reared mosquitoes fed 0.5% sucrose.

Consistent with our results, previous studies have demonstrated that a low sugar diet affects aspects of malaria vectorial capacity (the entomological components of R_0) in opposing ways (Gary and Foster, 2001; Braks et al., 2006; Stone et al., 2012; Junnila et al., 2015; Kessler et al., 2015; Yan et al., 2021; Tenywa et al., 2024). We found that a low sucrose diet increased the chance that mosquitoes were caught in traps (i.e. increased host seeking behaviours), likely because finding a food source is more critical for survival compared to well-resourced mosquitoes. While more host seeking causes greater contact between vectors and hosts, increasing transmission potential, we also observed lower overnight survival in the low-resourced mosquitoes. While survival was not quantified, whilst clearing mosquitoes from the semi-field system in the morning after the releases, we did not observe any live mosquitoes from the 0.5% sucrose group, but regularly encountered mosquitoes fed higher concentrations of sucrose and/or an additional blood meal in resting sites. Thus, low resources could curtail onward transmission if vectors do not survive long enough for malaria parasites to complete their development (Ohm et al., 2018). For mosquitoes with access to

blood meals but no sugar, laboratory studies suggest that the net outcome of the opposing impacts of increased host seeking and lower survival is a higher vectorial capacity (Gary and Foster, 2001). However, we have revealed that biting time of day is also modulated by mosquito nutritional status, further complicating the impact on malaria transmission, especially in areas with high ITN use. That low resourced mosquitoes host seek earlier in the evening (early biters) is consistent with the hypothesis that organisms shift to different temporal niches under low resource availability to maximise survival (Van Der Veen et al., 2017). Therefore, early biting in the evening rather than during the classical time window may be adaptive in regions with high ITN use because low-resourced mosquitoes are unlikely to have the energy reserves available to wait until humans leave bed nets in the morning. Therefore, to be able to assess the efficacy of ITNs, future studies need to integrate the impacts of resource availability on mosquito survival, biting rate, and biting time of day (Oke et al., 2022).

While better-resourced mosquitoes attempted to bite during the classical biting period as expected, they were also just as likely to be trapped in the morning (late biters). Such late biting by well-fed mosquitoes was unexpected but may be due to the lack of water/food sources provided in the semi-field enclosures, forcing untrapped mosquitoes to host seek to mitigate dehydration caused by rise in temperature and the reduction in humidity in the morning (Hagan et al., 2018; Lin et al., 2019). Alternatively, well-resourced mosquitoes which had failed to blood feed (i.e. had not entered a trap) during the night may have become resource-depleted during the night, leading to an increased propensity to continue host seeking at unusual times of day, like the low-resourced early biters caught in the evening. While our snapshot of wild mosquitoes had similar nutrient levels to low-resourced mosquitoes across the entire night, these mosquitoes were trapped after expending energy to host seek, and we found a suggestive trend for wild mosquitoes caught in the evening and morning to contain fewer lipids, further suggesting that resource availability modulates biting time of day. However, future studies with larger sample sizes and higher statistical power are needed to investigate these differences.

Biting in the morning has become more widespread across Africa since the introduction of ITNs (Akuoko et al., 2022; Sangbakembi-ngounou et al., 2022; Odero et al., 2024). For example, in Kenya, *An. gambiae* biting peaked in the night-time as expected, but also continued into the early morning hours after 4am (Odero et al., 2024). A similar pattern has been observed in Ghana, with the majority of biting (66%) occurring during the night-time, followed by approximately 20% occurring after 4am,

and the least biting (13%) occurring in the evening before 10pm (Akuoko et al., 2022), consistent with our field results. Biting time shifts do have a genetic component (Govella et al., 2023), and universal coverage of ITNs is approximately 75% in Ghana (Afagbedzi et al., 2023), so a late biting tendency could be an evasion strategy evolving in the mosquito population that we collected larvae from, rather than a plastic behavioural change.

Further work is needed to ascertain the extent to which early and late biting is genetically determined and/or due to behavioural plasticity. For example, mosquitoes (reared from wild-caught larvae) could be provided with ample nutritional resources throughout the night in a semi-field setting. Investigating whether they bite in the morning or wait until the next night after a failed attempt would tease apart whether the late biting is due to an evolved shift in biting time in response to ITN use, plastic responses due to resource depletion and/or dehydration, or a combination. Previous studies suggest behavioural plasticity is more influential than genetic changes (Govella et al., 2023), but the extent of genotype-by-environment (GxE) interactions has been overlooked. For example, early biting may only be adaptive in areas of high ITN use and low resource availability. Therefore, GxE interactions driven by resource availability could influence the evolution of biting time shifts. Modelling suggests that evolution of an early-biting phenotype is most likely to evolve in resource-poor areas with high ITN coverage (Stone et al., 2016). This is unsurprising because altering foraging rhythms usually has negative impacts (Gill et al., 2015), so shifts in activity have to provide a significant energetic benefit (Van Der Veen et al., 2017; Van Der Vinne et al., 2019). Alternatively, in areas of fluctuating resource availability, plastic mosquito genotypes that can switch between early or late dependent on their environmental conditions would have higher fitness (Pigliucci, 2005). Investigating the potential drivers (both genetic and environmental) for both early and late biting, and whether these are different, is key to being able to predict the success of current (and future) control tools targeting biting behaviour.

In addition to the impacts of nutritional resources on vectorial capacity via modulating the likelihood and time of day of host seeking, and affecting mosquito survival, resources also affect malaria parasite development in complex ways. For example, if early biters are more likely to be low-resourced mosquitoes, there are conflicting outcomes for parasites. While early biting is more suitable for parasite establishment in the mosquito due to lower night-time temperatures (Suh et al., 2020) and higher infectivity than in the day time (Rund et al., 2016; Pigeault et al., 2018; Schneider et al., 2018b), parasite development is slower and less successful in low-resourced

mosquitoes (Costa et al., 2018; Shaw et al., 2020; Habtewold et al., 2021). Furthermore, given that disruption to foraging rhythms reduces fitness in other insects (Gill et al., 2015), blood feeding at a non-classical time of day may exacerbate the negative impacts of resource limitation for parasites infecting early biting, relative to classical biting, vectors. While transmission at a non-classical time of day is clearly better for parasites than no transmission at all in areas of ITN use, recognising that transmission is time of day dependent is necessary for predicting epidemiology and parasite evolution. Future studies are needed to quantify how both nutritional resources and shifts in biting time of day alter both parasite- and mosquito-related components of vectorial capacity. These data also provide a foundation to assess how other factors affect malaria transmission, because different vector species vary in their foraging rhythms (Rund et al., 2016) and parasite susceptibility (Molina-Cruz et al., 2012), and mosquito age likely disrupts circadian rhythms (Ahmed et al., 2022). Overall, our results suggest that plasticity in biting time of day driven by variation in mosquito nutritional condition is an underappreciated contributor to residual malaria transmission. However, the overall impact on malaria transmission is difficult to predict, due to the potential for synergistic and antagonistic interactions between parameters contributing to parasite fitness (R_0).

Chapter 6. Testing a non-destructive assay to track *Plasmodium* sporozoites in mosquitoes over time

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6.1 Abstract

Background: The extrinsic incubation period (EIP), defined as the time it takes for malaria parasites in a mosquito to become infectious to a vertebrate host, is one of the most influential parameters for malaria transmission but remains poorly understood. The EIP is usually estimated by quantifying salivary gland sporozoites in subsets of mosquitoes, which requires terminal sampling. However, assays that allow repeated sampling of individual mosquitoes over time could provide better resolution of the EIP.

Methods: We tested a non-destructive assay to quantify sporozoites of two rodent malaria species, *Plasmodium chabaudi* and *Plasmodium berghei*, expelled throughout 24 h windows, from sugar-feeding substrates using quantitative PCR**.**

Results: The assay can quantify sporozoites from sugar-feeding substrates, but the prevalence of parasite positive substrates is low. Multiple methods to increase the detection of expelled parasites (running additional technical replicates; using groups rather than individual mosquitoes) did not increase the detection rate, suggesting that expulsion of sporozoites is variable and infrequent.

Conclusions: We reveal successful detection of expelled sporozoites from sugar-feeding substrates. However, investigations of the biological causes underlying the low detection rate of sporozoites (e.g., mosquito feeding behaviour, frequency of sporozoite expulsion, or sporozoite clumping) are needed to maximise the utility of using non-destructive assays to quantify sporozoite dynamics. Increasing detection rates will facilitate the detailed investigation on infection dynamics within mosquitoes, which is necessary to explain *Plasmodium's* highly variable EIP and to improve understanding of malaria transmission dynamics.

6.2 Background

Malaria, caused by *Plasmodium* parasites, (World Health Organization, 2020) is transmitted between vertebrate hosts by *Anopheline* mosquito vectors. Within the vector, parasites must mate, reproduce, traverse the midgut wall, replicate extensively and then migrate to the salivary glands. Only after all these processes (defined as sporogony) are completed, can parasites infect a new vertebrate host. The time it takes for parasites to complete their development in the vector (the extrinsic incubation period, EIP (Lefevre et al., 2017)) is usually reported to be 10-20 days (Ohm et al., 2018; Childs and Prosper, 2020). This is surprisingly long given that only a very small proportion of mosquitoes live longer than three weeks in the field (Clements and Paterson, 1981; Siria et al., 2022; Carrillo-Bustamante et al., 2023).

Small changes in the EIP can have a large effect on the number of mosquitoes living long enough to become infectious, making it a crucial parameter for transmission potential (i.e., R_0) (Ohm et al., 2018). Although the historical assumption that the EIP only depends on temperature (Nikolaev, 1935; Moshkovsky, 1946; Detinova, 1962) has been overturned (Ohm et al., 2018), understanding of other sources of variation in EIP remains limited. Variation in the EIP is associated with environmental factors, such as temperature and availability of the vector's resources, along with intrinsic differences between *Plasmodium* species. For example, *Plasmodium mexicanum*, vectored by the short-lived sand fly, has a shorter EIP (Lefevre et al., 2017), whereas *P. berghei* has a longer EIP, partly due to adaptation to the lower temperature of their vector's habitat (Vanderberg and Yoeli, 1966). In comparison, *P. chabaudi* and *P. falciparum* have similar development times, with the latter speeding up when mosquitoes receive an additional blood meal (Shaw et al., 2020). Furthermore, longer EIPs have been observed in *P. falciparum*-infected mosquitoes with lower salivary gland burdens (Andolina et al., 2024). Why malaria parasites cannot develop faster is a longstanding mystery and highlights the need to investigate whether constraints (such as the dynamics of resource availability within mosquitoes) and/or benefits to the parasite (such as transmission correlating positively with sporozoite number) shape the EIP (Carrillo-Bustamante et al., 2023).

Explaining the EIP is challenging because it is most commonly approximated as the time at which sporozoites are first visible in the salivary glands (Ohm et al., 2018). However, sporozoites may require a period of maturation to become infectious; heterogeneous gene expression suggests that not all sporozoites residing in the salivary glands are infectious (Bogale et al., 2021). Additionally, salivary gland

sporozoites may need to exceed a density threshold for onwards transmission to be likely. Transmission probability significantly increases above 10000 sporozoites for *P. yoelii* (Aleshnick et al., 2020), even though only tens to low hundreds of sporozoites are thought to be expelled during transmission (Rosenberg et al., 1990; Beier et al., 1992; Graumans et al., 2020; Andolina et al., 2024), but studies using *P. falciparum* suggest a lower (>1000) threshold (Churcher et al., 2017). Furthermore, some infected mosquitoes do not expel any sporozoites (Ponnudurai et al., 1991; Medica and Sinnis, 2005; Andolina et al., 2024) further complicating the correlation between salivary gland sporozoites and transmission probability.

Tools for estimating the EIP are also problematic; the EIP is typically estimated from terminal (i.e. destructive) sampling of a subset of mosquitoes from the population at intervals during sporogony. Sporozoites are usually assayed following dissection of the salivary glands for microscopic detection (Shapiro et al., 2017), or by molecular assays from (bisected) mosquitoes (Foley et al., 2012; Andolina et al., 2024). These methods have several limitations. First, terminal sampling prevents tracking individual mosquitoes over time, so EIP is estimated at population level. While population-level measures such as the median EIP (EIP_{50}) are useful for modelling purposes (Ohm et al., 2018), they do not consider the individual variation important for linking vector-parasite-environment interactions with the EIP and infectiousness (Guissou et al., 2021, 2023). Second, processing a subset of mosquitoes every few days is laborious and requires large numbers of infected mosquitoes.

Sporozoites are expelled during sugar feeding (Melanson et al., 2017; Ramírez et al., 2019) and expelled sporozoites have a greater chance of being infectious than those in the glands. Thus, using a non-destructive assay that quantifies expelled sporozoites on sugar-feeding substrates allows the infections of individual mosquitoes to be followed over time and can improve resolution of the EIP. Non-destructive sugar-based assays to quantify sporozoites, using PCR or immunoblotting detection of circumsporozoite protein, have been tested for groups or single mosquitoes infected with *P falciparum* (Golenda et al., 1992; Melanson et al., 2017; Brugman et al., 2018; Ramírez et al., 2019; Guissou et al., 2021), and for groups of *P. berghei*-infected mosquitoes (Billingsley et al., 1991; Brugman et al., 2018) with some success. While assays able to detect sporozoites from groups of mosquitoes are useful for field surveillance of malaria prevalence (Melanson et al., 2017; Brugman et al., 2018; Ramírez et al., 2019), assays sensitive enough to detect sporozoites from individual mosquitoes provide the best resolution of EIP and its determinants. Furthermore,

investigating the ecological and evolutionary determinants of the EIP, including the impact of host factors, requires model systems in which the full life cycle can be manipulated *in vivo*. Due to their tractability, rodent malarias are ideal, but there is no assay available for individual mosquitoes infected with these *Plasmodium* species. The most used model, *P. berghei*, is useful for proof of principle investigation of EIP-related questions, including onward transmission to a vertebrate host, but *P. chabaudi* provides a unique opportunity to investigate EIP at a similar parasite density and temperature (Spence et al., 2012) to *P. falciparum*.

Here we test a non-destructive method to detect *P. berghei* and *P. chabaudi* sporozoites from mosquitoes' sugar-feeding substrates. We compare how well this technique performs for *P. berghei* and *P. chabaudi* which have different optimal temperatures for sporogony and therefore different EIPs. We demonstrate that *Plasmodium* DNA from both species can be detected and quantified from sugar-feeding substrates. However, while the detection rate for sporozoites in mosquito expectorates is similar to other studies (Brugman et al., 2018; Guissou et al., 2021), parasite prevalence is low. We discuss potential explanations for low parasite prevalence in individual mosquito's expectorate and suggest further improvements to sugar-feeding assays.

6.3 Methods

The qPCR to quantify *Plasmodium* sporozoites was validated and used to determine the best sugar-feeding substrate for the assay, the range of sporozoite DNA concentrations that can be recovered from the substrates, as well as optimal storage conditions and sugar concentrations to minimise DNA degradation. Subsequently, the recovery of expelled sporozoites from individual mosquitoes was investigated, as well as methods to increase the detection of expelled parasites.

6.3.1 Mosquitoes and malaria infections

Anopheles stephensi SD500 mosquitoes were reared at 26°C, 70% relative humidity, in a 12L:12D h light cycle, with *ad libitum* access to 8% fructose solution post-emergence. Transmission to mosquitoes was achieved through blood-feeding mosquitoes on mice (8-10 week old male C57Bl/6) with microscopy-confirmed gametocytes of either *P. berghei* ANKA or *P. chabaudi* genotype ER (following (Spence et al., 2012; Birget et al., 2017)). Mosquitoes used for *P. berghei* transmissions were gradually acclimatised to 21°C prior to infectious blood feeds. All

mosquitoes were starved for 24 hours before infection and unfed females were removed on day 1 post-infectious blood meal (pIBM).

6.3.2 DNA extraction

DNA from microscopy-quantified blood stage parasites (Schneider et al., 2018b) was extracted from 5 µL blood using a semi-automatic Kingfisher Flex Magnetic Particle Processor and MagMAX^{TM}-96 DNA Multi-Sample Kit (ThermoFisher Scientific) as per (Schneider et al., 2018a), and was frozen at -20 °C until use. These blood-stage DNA samples were used to determine qPCR efficiency and the limit of detection (LOD).

DNA was extracted from head/thorax mosquito samples and feeding substrates following the CTAB-based phenol-chloroform extraction method from Chen *et al* (Chen et al., 2010) with minor modifications as described in Schneider *et al* (Schneider et al., 2018b). Extracted DNA was eluted in 30 µL (mosquitoes, supplemented feeding substrates) or 16 µL (mosquito expectorate substrates) water, and frozen at -20 °C until use. Mosquito, but not feeding substrate DNA extracts, were diluted 4-fold to reduce the effect of inhibitors originating from mosquito material on the performance of the PCR. Seven microliters of (diluted) DNA extracts were used in all PCR reactions, and data are presented as genomes/PCR, unless stated otherwise, to account for differences in sample processing.

6.3.3 Quantification of Plasmodium by quantitative PCR

Both *P. berghei* and *P. chabaudi* were assayed by a quantitative PCR (qPCR) targeting a region of the 18S rRNA gene that is highly conserved among *Plasmodium* species (Bell et al., 2009). Parasite genomes were quantified by comparing threshold cycle (Ct) against a standard curve, generated from DNA extracted from blood stage parasites of either *P. berghei* ANKA or *P. chabaudi* genotype ER (see “DNA extraction”). Due to extraction efficiency differences between blood and mosquito/substrate extractions, absolute numbers of sporozoites may be slightly overestimated but relative differences between experimental groups will remain the same. Negative water controls were included to identify false positives.

6.3.4 Optimising the assay

The assay was optimised using two reference DNA samples from sporozoite-infected mosquitoes. DNA samples from *Plasmodium*-infected mosquitoes, shown by qPCR to have high sporozoite loads were pooled to create one reference DNA sample for

P. berghei (2967 genomes/µL) and one for *P. chabaudi* (5099 genomes/µL). These reference samples were used to determine: 1) which type of feeding substrate type returned an optimal DNA yield, and whether 2) DNA could be detected across a range of concentrations, 3) DNA degradation occurred during the collection period, and 4) sugar content impacted DNA yield.

The most suitable feeding substrate was selected by comparing the recovery of parasite DNA from 15 mg cotton wool, a 1cm^2 cotton pad (Boots UK) or a 1cm^2 filter paper (Whatman No. 1). Each substrate (n=3 per substrate type) was soaked in 8% fructose, supplemented with 5 µL *P. berghei* or *P. chabaudi* reference DNA and stored at 26 °C and 70% relative humidity for 24 h to mimic housing conditions for *P. chabaudi*-infected mosquitoes. Reduced DNA yields are expected at higher temperatures (Bulla et al., 2016), so these conditions provide a conservative estimate of assay performance for *P. berghei*. DNA yield was calculated by comparing qPCR results directly from reference DNA with those from spiked feeding substrates, accounting for any dilutions during sample processing. Subsequent tests were conducted using cotton wool, and all substrates were kept in the same conditions as described above. Second, to confirm that DNA could be consistently detected and quantified across a range of concentrations, cotton wool substrates were supplemented with 5 µL of serial dilutions of *P. berghei* (neat 10^0 to $5x10^{-3}$ dilution) or *P. chabaudi* (neat 10^0 to $5x10^{-4}$ dilution) reference samples, (n=3 per dilution/species), immediately after soaking in 8% fructose (time point 0 h). Linearity of quantification and the limit of quantification (LOQ), relative to the limit of detection (LOD), was quantified. Third, DNA degradation under conditions mimicking mosquito housing was tested by comparing DNA recovery from cotton wool supplemented with 5 µL reference sample (10^0 to 10^{-2} dilution for each species) either immediately after soaking in 8% fructose (time point 0 h) or at collection (time point 24 h) (n=3 per time point/species). Finally, the impact of sugar concentration on DNA yield was tested by soaking cotton wool substrates in distilled water, 1% or 8% fructose and supplemented with 5 µL of serial dilutions of *P. berghei* or *P. chabaudi* reference samples (neat 10^0 to 10^{-2} dilution, n=3 per dilution/species). Parasite DNA recovery was compared between the 3 sugar concentrations.

6.3.5 Testing the assay on mosquito expectorate samples

To collect expectorate samples, mosquitoes were moved to paper cups, either individually (*P. berghei* n=13; *P. chabaudi* n=10) or in groups (*P. berghei,* 4 mosquitoes/cup, n=5 cups). To increase the likelihood of sugar feeding, mosquitoes

were starved for 24 h prior to being provided with a feeding substrate, which was collected 24 h later and stored at -20 °C until DNA extraction. This 2-day starvation–feeding cycle was repeated twice during days 22-25 pIBM for *P. berghei*, and days 12-15 pIBM for *P. chabaudi* (Figure 6.1). After both sets of expectorate samples were collected, mosquitoes were anaesthetised on ice and bisected following Foley *et al* (Foley et al., 2012). Head-thorax specimens were stored at -20 °C until DNA extraction and subsequent salivary gland sporozoite quantification by qPCR. Only data from sporozoite-infected mosquitoes that survived for the entire experiment were included in all analyses (n=1 *P. berghei* and n=1 *P. chabaudi* uninfected individual mosquitoes were excluded; no uninfected mosquito groups were detected).

6.3.6 Statistical analysis

Data analyses were performed using R v. 4.1.3. Linear models were used to determine PCR efficiency and compare this between species. The absolute limit of detection (LOD) (Bustin et al., 2009), defined as the minimum concentration that can be detected with a sensitivity of 100%, was determined using plateau-linear models fitted to qPCR Ct values and associated genome counts (SSplin, *nlraa* package (Miguez, 2022)). These models predict the switching point from a plateau to a linear slope, thus indicating when the qPCR true positivity rate dropped below one. Parasite densities below the LOD were set to zero. To determine the most suitable substrate and sugar concentration, and test for DNA degradation over time, linear models were used to investigate the effect of the variable tested (substrate, sugar, or time), parasites species, DNA concentration (if relevant), and all their interactions on Ct value. DNA yield across *Plasmodium* concentrations was analysed using linear models for *P. chabaudi* and the SSplin function for *P. berghei*, for which this non-linear model fitted better than a linear regression (ΔAICc > 2).

The presence/absence of parasite DNA from expelled sporozoites over time was tested using binomial generalised linear models (glm), including an interaction between *Plasmodium* species and salivary gland burden. Further binomial glms were used to test whether processing a larger proportion of the mosquito expectorate DNA extract (summing parasite densities detected in two qPCR replicates) or collecting expectorates from small groups of *P. berghei*-infected mosquitoes rather than individuals improved detection rates, including species and either replicate or grouping, as well as their interaction into the models. Negative binomial models (glm.nb function, *MASS* package (Venables and Ripley, 2002)) were used to investigate whether the number of expelled sporozoites on positive substrates was

affected by (1) day and salivary gland burden, and how this varied by species; (2) summing parasite densities from two qPCR replicates, by species; (3) grouping *P. berghei*-infected mosquitoes, by day; and (4) whether salivary gland sporozoite burden differed between species.

Models were minimised using likelihood ratio tests, and AICc for non-nested models. All models met model assumptions, confirmed by simulating and plotting residuals using the *DHARMa* package (Hartig, 2022). Confidence intervals were obtained from statistical models or, in the case of confidence intervals for quotients, using Fieller's method (Fieller, 1940).

6.3.7 Ethics statement

All procedures comply with the UK Home Office regulations (Animals Scientific Procedures Act 1986; SI 2012/3039) and were approved by the ethical review panel at the University of Edinburgh (PPL PP8390310).

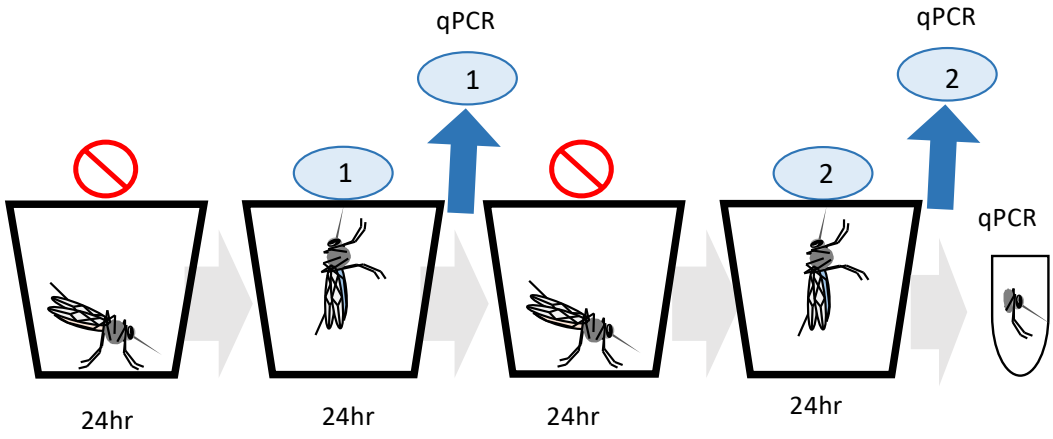

Figure 6.1. Cotton substrate collection timings from *Plasmodium* infected mosquitoes. Mosquitoes were moved to paper cups and starved for 24 h, then provided access to a sugar-feeding substrate. After 24 h, the substrate was collected for sporozoite detection by qPCR. This cycle was repeated twice, such that two substrates were collected per mosquito. The cycle started on day 22 pIBM for *P. berghei* and day 12 pIBM for *P. chabaudi*.

6.4 Results

6.4.1 Validation of qPCR for sporozoite detection: True and false positivity

The qPCR assay targeting the 18S rRNA gene has been previously validated for sporozoite detection, achieving a 95% amplification efficiency and a limit of detection of <10 parasites/PCR reaction (Bell et al., 2009). We replicate this high qPCR performance using DNA extracted from blood stages of *P. berghei* (0.5 to 6428 genomes/PCR reaction) or *P. chabaudi* (0.2 to 75461 genomes/PCR reaction), achieving an amplification efficiency of 99.5±2.6%, R^2=0.99, with equal performance between species (log_{10} parasite density by species interaction: $F_{1,20}$=0.51, p=0.48). Although quantification is accurate when low parasite densities are detected, detection rates drop at lower densities. The limit of detection (LOD, the concentration at which the true positivity rate drops below 1) was 4.4 genomes/PCR reaction (Ct 36.7±0.5) for *P. berghei* (Figure 6.2A) and 0.8 genomes/PCR reaction (Ct 38.4±0.1) for *P. chabaudi* (Figure 6.2B). At parasite densities below the LOD (i.e., higher Ct values), the rate of false negatives increases and these densities were set to zero. False positives (water samples) were not detected at concentrations above the LOD for either species.

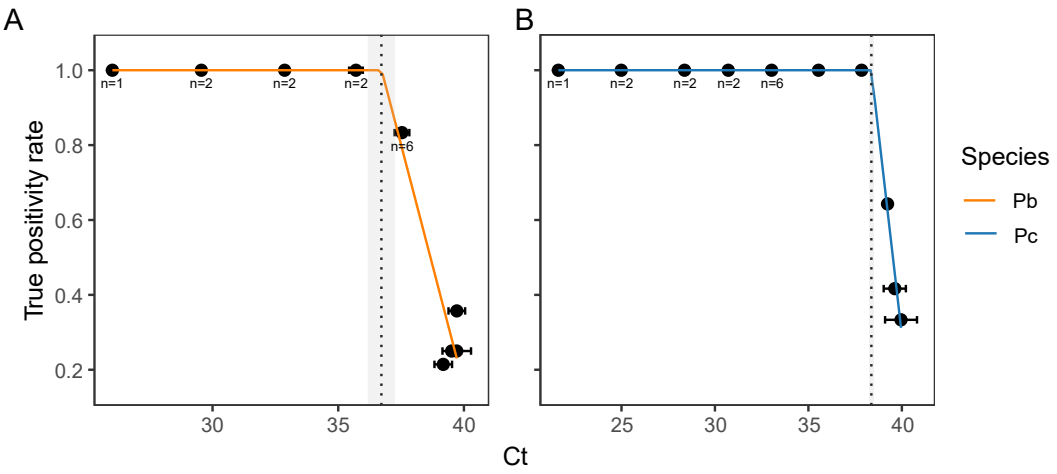

Figure 6.2. True positivity rates, determined from quantification of a serial dilution of DNA from blood-stage parasites for *P. berghei* (A, orange) and *P. chabaudi* (B, blue). Mean Ct values ±SEM are presented for *P. berghei* (0.001 to 6428 genomes/PCR reaction) and *P. chabaudi* (0.05 to 75461 genomes/PCR), tested in n=12 replicates unless stated otherwise in the graph. The limit of detection (LOD, the concentration at which the true positivity rate drops below 1) ±SEM, predicted using a plateau-linear function, is 4.4 (Ct 36.7±0.5) or 0.8 (Ct 38.4±0.1) genomes/PCR reaction for *P. berghei* and *P. chabaudi*, respectively (dotted lines ± shading).

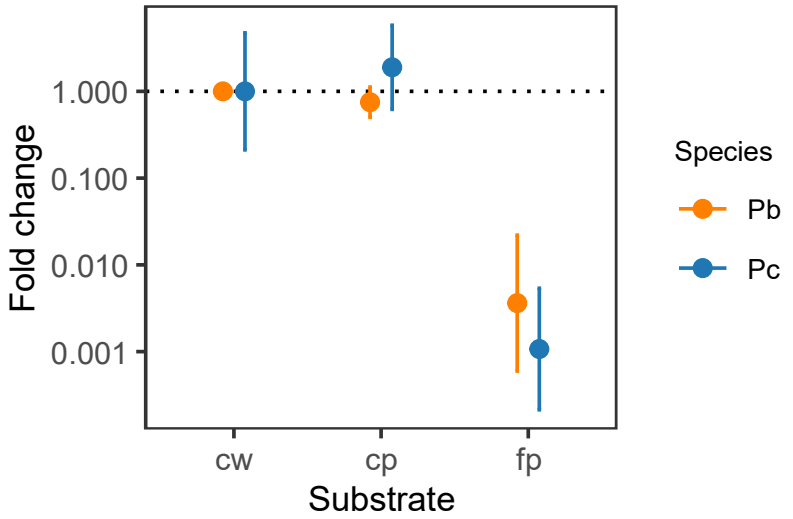

Figure 6.3. Relative DNA yield from substrates soaked in 8% fructose and supplemented with reference DNA extracted from *P. berghei* (orange) or *P. chabaudi* (blue) infected mosquitoes. DNA yield is presented as mean fold difference ± 95% CI (n=3/substrates/species) relative to the mean DNA yield for cotton wool for each species and displayed on a $\log_{10}$ scale to clearly visualise both increased and decreased DNA yield. Cotton wool (cw); cotton pads (cp), filter paper (fp).

6.4.2 Optimising the assay

To determine the most suitable feeding substrate for the assay, three different substrates, soaked in 8% fructose and supplemented with 5 µL reference DNA from *P. berghei* or *P. chabaudi* infected mosquitoes, were tested: filter paper, cotton wool and cotton pads. DNA yield varied by substrate type and parasite species (substrate by species interaction: $F_{2,12}$=4.42, p=0.036). Compared to cotton wool extraction efficiencies (21% for *P. chabaudi*, 25% for *P. berghei*), cotton pads resulted similar DNA yields, while filter paper yielded 933-fold (CI: 186-4683) and 276-fold (95% CI: 45-1675) lower DNA for *P. chabaudi* and *P. berghei* respectively (Figure 6.3). Based on DNA yield, and ease of use, cotton wool was selected as the feeding substrate for the remainder of this study.

Assay performance for cotton wool was tested by supplementing cotton wool substrates with 5 µL of a serial dilution of reference DNA for *P. berghei* (17-3461 genomes/PCR) or *P. chabaudi* (3-5949 genomes/PCR) (Figure 6.4). Non-linearity for *P. berghei* samples with Ct > 36.5±0.4 shows that quantification became inaccurate at parasite densities below 50 genomes/PCR. This switching point is referred to as the limit of quantification (LOQ) and occurs at a similar Ct value as for *P. berghei* blood samples (36.7±0.5; dotted line, Figure 6.4A). For *P. chabaudi*, the linear dynamic range covered all tested parasite densities, suggesting that we can confidently detect and quantify *P. chabaudi* genomes from cotton wool substrates up until the LOD as determined by the *P. chabaudi* blood samples above (Ct 38.4±0.1; Figure 6.4B). The slopes in Figure 6.4 were steeper than expected, indicating PCR efficiencies of 70.4±8.9% for *P. berghei* and 78.2±4.5% for *P. chabaudi*, which could be explained by covering a wider range of DNA concentrations: DNA quantities were underestimated for low density samples, with Ct values at/above the LOD. To maximise chances of mosquitoes feeding and expelling sporozoites, access to substrates lasted for 24 h. Because the conditions in which mosquitoes are kept may not be optimal to preserve DNA, we investigated DNA degradation over 24 h. Specifically, we compared DNA yield from cotton wool supplemented with DNA at the time of sugar soaking (time point 0) or supplemented at the time of collection 24 h later (time point 24). As expected, lower DNA concentrations result in a lower DNA yield (DNA: $F_{1,33}$=2036.6, p<0.001). While the absolute number of genomes varies between species, reflecting the higher parasite densities in the *P. chabaudi* compared to the *P. berghei* reference sample (species: $F_{1,33}$=310.9, p<0.001), quantification is equally efficient in both species (DNA by species interaction: $F_{1,31}$=0.04, p=0.87). We did not observe DNA degradation after 24 h of storage for either species (time by species interaction: $F_{1,29}$=0.0004, p=0.98; time: $F_{1,32}$=3.71, p=0.063), across all parasite densities (time by DNA by species interaction: $F_{1,29}$=1.76, p=0.20; time by DNA interaction: $F_{1,31}$=0.11, p=0.75) (Figure 6.5A).

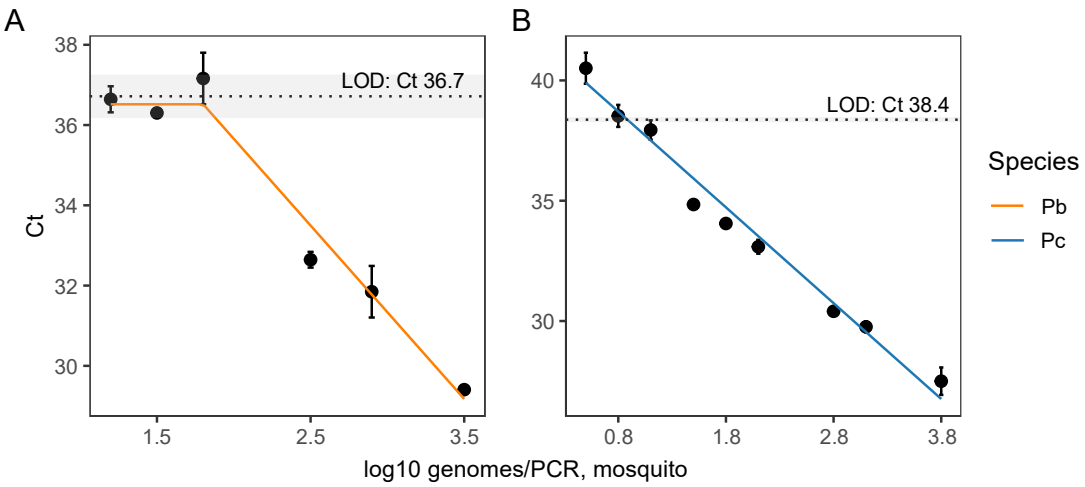

Figure 6.4. Linearity of quantification for *P. berghei* (orange; A) and *P. chabaudi* (blue; B) reference DNA (cotton wool, 8% fructose). Mean Ct values ± SEM are presented for reference DNA originating from infected mosquitoes (log_{10} genomes/PCR, mosquito) ranging from 17-3461 (*P. berghei*) or 3-5949 (*P. chabaudi*) genomes/PCR (n=3/concentration/species). The limit of quantification (LOQ, Ct at which samples can be detected but quantification becomes inaccurate) is determined as the switch point where the plateau ends. The dashed line shows the Ct value associated with the limit of detection ± SEM (LOD, see Figure 6.2) for blood samples.

Mosquito feeding substrates have a high sugar concentration (usually fructose or glucose), which may affect extraction efficiency and subsequent DNA amplification. To test whether substrate sugar content affected DNA yield, we compared DNA recovery from cotton wool substrates soaked in 0, 1 or 8% (w/v) fructose, and supplemented with reference DNA at time point 0. Our analysis confirmed higher parasite densities in the *P. chabaudi*, compared to *P. berghei* reference sample (species: $F_{1,49}$=80.4, p<0.001), that lower DNA concentrations result in lower DNA yields (DNA: $F_{1,49}$=639.0, p<0.001), and that quantification of DNA is equally efficient for both species (DNA by species interaction: $F_{1,48}$=0.84, p=0.36). Sugar concentration impacts DNA yield (sugar: $F_{2,49}$=8.10, p<0.001); lower concentrations reduce the yield compared to 8% fructose by 1.5-fold (95% CI: 1.1-2.1) for 1% and 2.4-fold (95% CI: 1.7-3.3) for 0% (Figure 6.5B), in the same manners across parasite species and densities (sugar by DNA by species interaction: $F_{2,42}$=0.56, p=0.58; sugar by species interaction: $F_{2,44}$=0.13, p=0.88; sugar by DNA interaction: $F_{2,46}$=1.30, p=0.28). Together, these results confirm that collecting mosquito expectorate over a period of 24 hours on substrates soaked in 8% fructose is optimal.

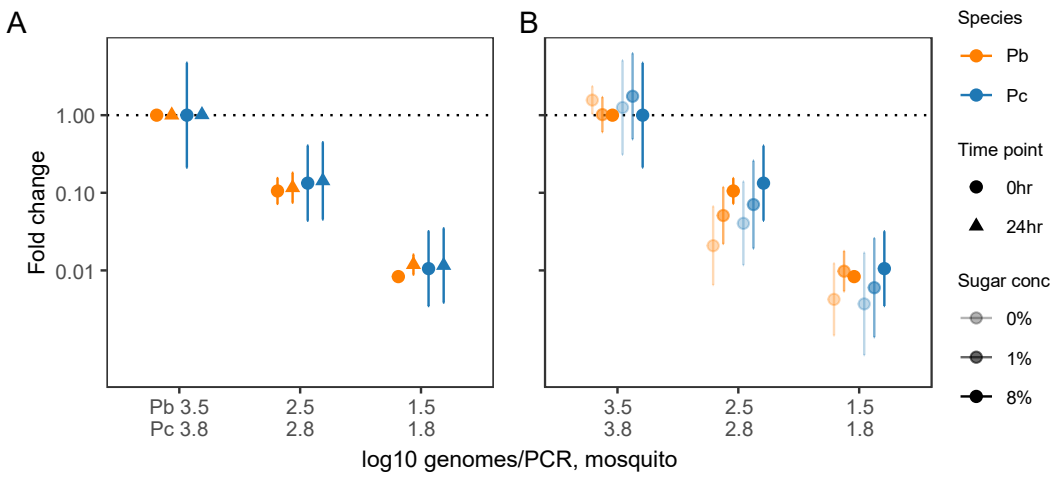

Figure 6.5. Relative DNA yield in response to storage time (A) and sugar concentration (B) for reference DNA quantified from cotton wool substrates. Data points present mean fold change ± 95% CI, relative to substrates supplemented with neat reference DNA at time point 0 (A), or soaked in 8% fructose (B), for each species. Dilutions of reference DNA, originating from infected mosquitoes (log_{10} genomes/PCR, mosq), range from 35-3461 (*P. berghei*, orange) or 59-5949 (*P. chabaudi*, blue) genomes/PCR (n=3 /concentration/group).

6.4.3 Testing the assay using mosquito expectorate samples

Following optimisation using reference DNA, we tested the assay's performance using mosquito expectorate samples. We allowed *Plasmodium*-infected mosquitoes, housed individually (n=12 *P. berghei*, n=9 *P. chabaudi*) or in small groups (n=5 groups of 4 mosquitoes/group for *P. berghei*), to feed for 24 hours on cotton wool substrates soaked in 8% fructose, which were collected twice per (group of) mosquito(es). We compared the prevalence and density of parasite DNA in the feeding substrates between species and by group size.

There was no correlation between the number of sporozoites in the salivary glands of individual mosquitoes and the number of expelled parasites on positive feeding substrates (salivary gland burden: χ^2_1=0.01, p=0.92) for either species (salivary gland burden by species interaction: χ^2_1=0.84, p=0.36). However, we detected 11-fold (95% CI: 3.6-34.9) more expelled parasites on positive feeding substrates for *P. berghei* compared to *P. chabaudi* (species: χ^2_1=10.7, p=0.001; Figure 6.6A). This likely reflects the 3.4-fold (95% CI: 1.2-9.6) higher sporozoite burden in the salivary glands for *P. berghei* compared to *P. chabaudi*-infected mosquitoes (species: χ^2_1=4.50, p=0.03; Figure 6.6B). The number of sporozoites expelled was 2.9-fold (95% CI: 1.3-6.3) higher on the first vs. second substrate collection day (day: $\chi^2_{(1)}$=4.8, *P*=0.03). This may be due to a higher representation of *P. berghei*-infected mosquitoes, with higher sporozoite burdens, in positive substrates of the first (3/4: 75%) vs second collection day (4/6: 67%) (Table 6.1).

Table 6.1. Parasite detection rates from sugar feeding substrates, fed on by individual or groups of mosquitoes.

	P. berghei (days 23, 25 pIBM)			***P. chabaudi*** (days 13, 15 pIBM)		
	Det. Rate [a]	**Ct [b]**	**Sporozoites[b]**	**Det. rate [a]**	**Ct [b]**	**Sporozoites[b]**
Individuals						
1st Substrate	0.25 (3/12)	33.6±0.6	47.4±16.5	0.11 (1/9)	37.9	1.5
2nd Substrate	0.33 (4/12)	35.2±0.5	13.3±4.0	0.22 (2/9)	37.5±0.8	2.2±1.1
Groups						
1st Substrate	0.40 (2/5)	33.0±2.3	172±160	n.d.	n.d.	n.d.
2nd Substrate	0.60 (3/5)	34.6±1.2	34.4±21.8	n.d.	n.d.	n.d.

[a] proportion of positive substrates (number positive/total tested). [b] mean Ct value or number of sporozoites/PCR with SEM for positive substrates. n.d : not done

While 50% (6/12) of *P. berghei*- and 33% (3/9) of *P. chabaudi*-infected mosquitoes (confirmed to be positive for salivary gland sporozoites via qPCR) generated at least one positive substrate, the overall proportion of positive feeding substrates from individual mosquitoes was low at 29% (7/24) for *P. berghei* and 17% (3/18) for *P. chabaudi*. Only one mosquito, infected with *P. berghei*, returned a positive substrate on both collection days, and the number of positive substrates was similar across

days for both species (day: χ^2_1=0.63, p=0.43) (Table 6.1). As expected, the probability of detecting parasites on feeding substrates increased with increasing salivary gland burden (χ^2_1=5.92, p=0.015). This was regardless of species, suggesting that parasites from *P. berghei* and *P. chabaudi*, after adjusting for parasite density, are equally well detected in feeding substrates (salivary gland burden by species interaction: χ^2_1=0.30, p=0.58; species: χ^2_1=0.004, p=0.95).

To investigate whether detection rates of parasites on feeding substrates could be improved, we doubled the proportion of the DNA extract quantified by qPCR from 0.4 to 0.9, by running a replicate qPCR reaction. Doubling the volume of sample tested did increase the density of parasites detected across both replicates in positive cotton substrates by 2-fold (95% CI: 1.0-5.1) (replicate: χ^2_1=3.95, p=0.047, Figure 6.6C), regardless of species (species by replicate interaction: χ^2_1=0.06, p=0.81). However, for each species, the proportion of feeding substrates from which parasite DNA was detected was identical (*P. berghei* 7/24, *P. chabaudi* 3/18). Subsequently, we tested if housing mosquitoes in small groups (n=4 mosquitoes) could increase the amount of DNA per feeding substrate (thus improving detection rates), whilst preserving the possibility to obtain data from replicate groups and track results over time. We used *P. berghei*, because the higher density of sporozoites in expectorates increases the likelihood that expectorates of multiple mosquitoes will contain detectable sporozoites. As expected, grouping increased the number of expelled sporozoites by 3-fold (95% CI: 1.1-8.5) (grouping: χ^2_1=4.00, p=0.045; Figure 6.6D). Less sporozoites were expelled on the second collection day, regardless of group size (day by grouping interaction: χ^2_1=0.11, p=0.74, day: χ^2_1=6.14, p=0.013). However, we did not detect an increase in the rate of detection of *P. berghei* for grouped mosquitoes (grouping: χ^2_1=1.31, p=0.25): 50% (5/10) of cotton substrates obtained from groups returned positive expectorate samples, compared to 29.2% (7/24) from individually-housed mosquitoes (Table 6.1).

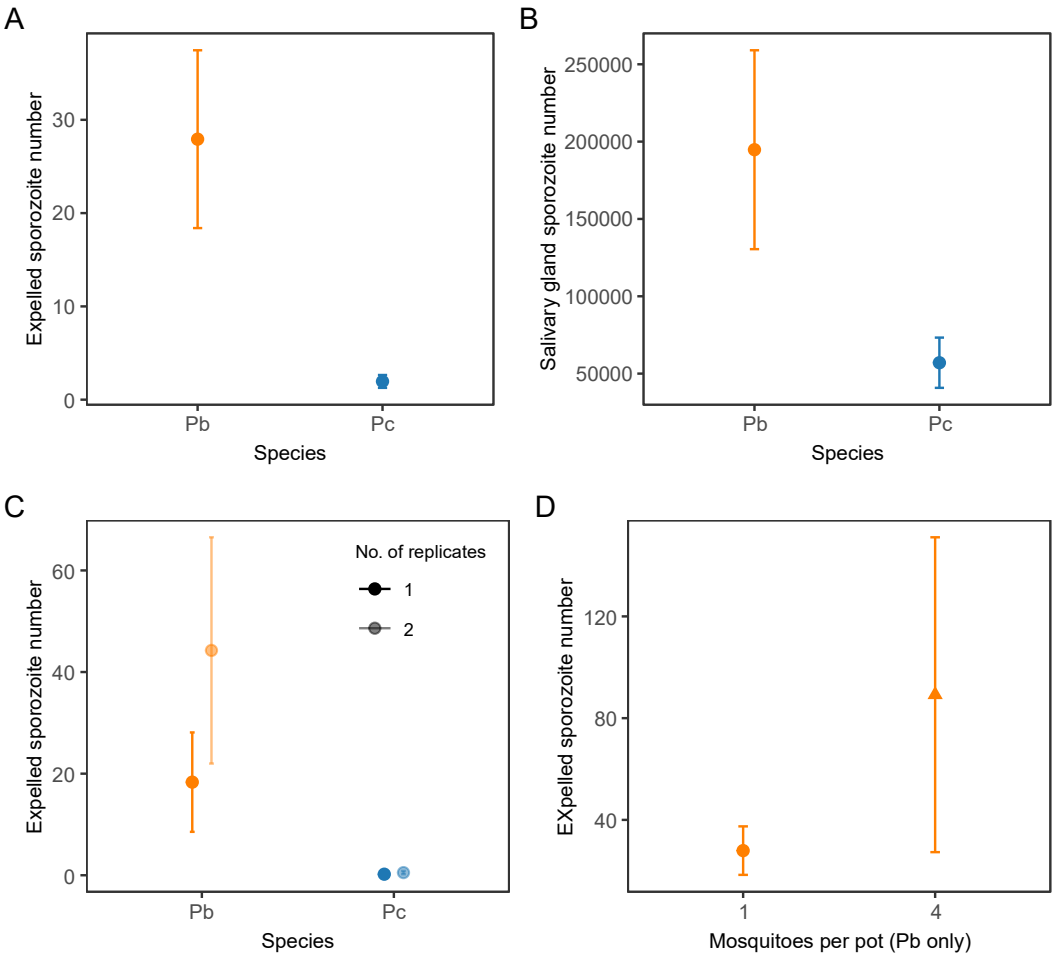

Figure 6.6. Mean sporozoite densities ± SEM for sporozoite-positive expectorates (A,C,D; per PCR) and salivary glands (B; per mosquito) for mosquitoes infected with *P. berghei* (orange) and *P. chabaudi* (blue). Data are presented for single (dark colours) or double (C, light colour) qPCR replicates, and for individual mosquitoes (circles) or groups of 4 mosquitoes (triangles, D).

6.5 Discussion

We tested a non-destructive assay for detecting and quantifying sporozoites from mosquito expectorate. Like previous studies (Melanson et al., 2017; Brugman et al., 2018; Ramírez et al., 2019; Guissou et al., 2021), we demonstrate that DNA from *Plasmodium* sporozoites can be detected from feeding substrates. Moreover, our assay detects rodent *Plasmodium* DNA across a range of concentrations, with no evidence of DNA degradation over 24 h of sample collection, and optimal DNA yield using 8% fructose commonly used to maintain lab mosquitoes. However, when testing

individual mosquito expectorates (as opposed to reference DNA) the proportion of positive substrates was low.

Our study differs from previous studies in that we investigated individual mosquitoes infected with two commonly used rodent laboratory models, *P. berghei* and *P. chabaudi*. The assay performed similarly for both species, but expectorates from *P. berghei*-infected mosquitoes contained 11-fold more sporozoites than *P. chabaudi*-infected mosquitoes. This is not unexpected considering that *P. berghei* generally reaches higher oocyst and sporozoite densities (Blanford et al., 2009; Spence et al., 2012; Pollitt et al., 2013), and there may be malaria species-specific differences in sporozoite inoculum size. Indeed, more sporozoites are needed to successfully initiate infections in vertebrate hosts for *P. berghei* compared to *P. yoelii,* suggesting that per-sporozoite infectivity is lower for *P. berghei* (Weiss, 1990; Briones et al., 1996; Medica and Sinnis, 2005; Jin et al., 2007). We confirm previous reports (Guissou et al., 2021, 2023) that the rate of detection for sporozoite expulsion (prevalence) increases with higher salivary gland burdens, but different from Andolina *et al* (Andolina et al., 2024) we, and others (Beier et al., 1991a, 1991b; Ponnudurai et al., 1991; Walk et al., 2018) find no correlation between the number of expelled sporozoites (density) and salivary gland burdens. Therefore, like previous studies using *P. falciparum* (Churcher et al., 2017; Guissou et al., 2021, 2023), our data are consistent with the hypothesis that mosquitoes with higher salivary gland burdens are more likely to expel sporozoites and transmit malaria.

Low detection rates of expelled sporozoites could be due to technical limitations of the assay. The qPCR limit of detection of four and one genome(s) per PCR for *P. berghei* and *P. chabaudi* respectively, is equivalent to 68 *P. berghei* or 14 *P. chabaudi* genomes (i.e. sporozoites) per substrate when taking into account sample processing and DNA recovery. Additionally, proteins present in mosquito saliva can interact with sporozoites (Schleicher et al., 2018), potentially reducing the stability of expelled sporozoites which could raise the detection threshold. Therefore, low densities of expelled sporozoites may have gone undetected. However, detection rates did not improve by running multiple technical replicates, nor for expectorates from groups of four *P. berghei*-infected mosquitoes. Instead, it is more likely that not all substrates contain sporozoites. Similar low prevalence of positive feeding substrates was shown in Brugman *et al* (Brugman et al., 2018) where 31% (day 21 pIBM) and 55% (day 23 pIBM) of cotton wool DNA extracts were positive for groups of three *P. berghei*-infected mosquitoes, in comparison to 40% (day 23 pIBM) and 60% (day 25 pIBM) for

our mosquito groups. Depending on the mosquito species used, 8-52% of feeding substrates contained DNA of the human malaria parasite *P. falciparum* (Guissou et al., 2021). Our values of 29% and 17% of total positive substrates collected for individually housed *P. berghei* and *P. chabaudi*-infected mosquitoes respectively sit within this range. In addition, the proportion of individually housed *An. stephensi* mosquitoes generating at least one positive substrate over 24 h (33%, *P. chabaudi* and 50%, *P. berghei*) is within the range observed for *P. falciparum* (35%, FTA cards (Melanson et al., 2017); 61%, artificial skin (Andolina et al., 2024)). While higher proportions of mosquitoes with at least one *P. falciparum* positive cotton substrate (93%) were reported in Guissou *et al* (Guissou et al., 2021), up to ten substrates per mosquito were collected, thus increasing the chance of at least one substrate being positive. Together these data indicate that the low detection rate of parasites on feeding substrates may be common.

Instead, low detection rates on feeding substrates could be explained by mosquito feeding behaviour, sporozoite biology or a combination of both. While mosquitoes do not expel sporozoites every day (Brugman et al., 2018; Guissou et al., 2021, 2023), female mosquitoes are likely to sugar-feed daily especially if they do not have access to a blood meal (Gary and Foster, 2006). As mosquitoes in our study were starved for 24 hours in between access to substrates to increase feeding rate, a lack of sugar-feeding is unlikely to explain the absence of sporozoites on the feeding substrates. It is generally assumed that mosquitoes salivate in a similar way during sugar- and blood-feeding (Billingsley et al., 1991; Melanson et al., 2017), although different enzymes are released from different lobes during the two types of feeding, and thus sporozoite expulsion may vary too. Furthermore, sporozoite clumping (Li et al., 1992; Frischknecht et al., 2004) could increase variation in expulsion probability and numbers of expelled sporozoites, as observed for *P. yoelii*–infected mosquitoes (Medica and Sinnis, 2005). *P. berghei* sporozoites were not detected from 26 days pIBM onwards (Brugman et al., 2018), and we observed lower *P. berghei* sporozoite expulsion on day 25 compared to 23 pIBM, , suggesting that sporozoites may degenerate (Barber, 1936) or deplete (Porter et al., 1954) over time. If so, *Plasmodium* species may vary in sporozoite lifespan in the glands; for example, *P. falciparum* sporozoites have been shown to be expelled for several weeks (Guissou et al., 2021).

How the quantity and quality of sporozoites (both in the salivary glands and expectorate) influences the probability of transmission remains mysterious. While our

assay does not test for infectivity of expelled sporozoites, expelled sporozoites are transcriptionally different to those in the glands (Bogale et al., 2021), and thus may vary in their properties, including infectivity. Therefore, a more appropriate measure of EIP may be the time at which sporozoites are first expelled, rather than when sporozoites appear in the salivary glands, highlighting the need for sensitive non-destructive assays to determine the dynamics of sporozoite expulsion over time. Ideally, the frequency of expulsion should also be accounted for when estimating infectivity throughout a mosquito's lifespan. While our assay can detect expelled sporozoites from sugar feeding substrates for two rodent malaria species, like other currently available assays for *P. falciparum* and *P. berghei*, further improvements are needed to track EIP over time in individual mosquitoes. Identifying why detection rates are low remains a key challenge for improving the assay. Our qPCR assay has high sensitivity, so increasing DNA recovery (e.g. a liquid-only feeding system could improve DNA extraction efficiency) is most likely to improve detection rate. Additionally, the frequency of sugar feeding could be monitored by video, or by supplementing sugar with food colouring. Confirming how often mosquitoes feed would allow untouched negative substrates to be excluded and is also key to resolving the likelihood of sporozoite expulsion over time.

6.6 Conclusions

Rodent malaria species are a valuable laboratory tool for comparison between different *Plasmodium* species and for asking broad questions about *Plasmodium* biology. We show that expelled sporozoites from two different rodent malaria species can be detected from feeding substrates, but further improvement is needed to use this assay for tracking sporozoite expulsion from individual mosquitoes. The low rate of parasite detection in feeding substrates suggests that the appearance and burden of salivary gland sporozoites may not be the most appropriate measure of mosquito infectivity, and that the definition of EIP may require updating. Tracking expelled sporozoites in individual mosquitoes, rather than using salivary gland sporozoite dissections, would be optimal, whilst facilitating studies to identify how environment-parasite-vector interactions influence EIP and infectivity to vertebrate hosts over time.

Chapter 7. General Discussion

This thesis aimed to integrate evolutionary ecology, parasitology and vector biology to investigate the sources of variation that shape within-vector parasite development and onward transmission. Specifically, I have used a mosquito-malaria parasite system to (i) develop a framework for investigating the agents of selection imposed on parasites as a consequence of vector control tool use, (ii) reveal that parasite genotypes differ in within-vector traits that impact onward transmission potential, (iii) demonstrate that parasites modulate their within-vector growth in response to nutritional resource availability in ways that appear to maximise fitness, (iv) identify mosquito nutritional status as a driver of temporal shifts in vector foraging behaviour, (v) examine the implications for parasite transmission in the context of vector control tools, and (vi) develop and test a novel assay to track onwards transmission over time. In this chapter, I summarise my results and their broader context to develop future research directions, and suggest translational applications of my findings.

7.1 Adopting an evolutionary ecology approach to explain parasite evolution

Despite the widespread use of insecticide-based vector control tools (VCTs) contributing to the evolution of insecticide resistance and evasion strategies in mosquito populations, the impact of VCT-induced changes on parasite evolution has been overlooked. In Chapter 2, I address this by considering how mosquito responses to VCTs impact on parasite ecology and derive an eco-evolutionary framework for predicting parasite responses. Specifically, I collated the impacts of VCTs into overarching agents of selection on parasites and discuss the ways that parasites may evolve to maintain fitness. While predicting and monitoring parasite responses to VCTs (and other environmental changes) is a huge and multidisciplinary challenge, I suggest where to start to make progress with the key open questions. For example, I highlight the importance of understanding the extent of heritable genetic variation, phenotypic plasticity and genotype-by-environment interactions (GxE) for parasite traits exposed to selection as a result of VCT use. This is because the evolutionary potential of parasite populations relies on these non-mutually exclusive processes, which can shape the direction and strength of their responses (Oke et al., 2022). In addition, I also emphasise the need to investigate how changes to vector biology as a consequence of vector control can directly and/or indirectly affect parasite fitness, and the need for better methods to measure parasite transmission traits.

In my thesis, I did not explicitly investigate the impact of VCTs on parasite fitness, because there is currently a lack of consensus on how VCTs influence parasite ecology, and there is a more urgent need to focus explicitly on testing fundamental evolutionary predictions using proof-of-concept studies. This is because variation in within-vector parasite traits has been overlooked (Lefevre et al., 2017), and it is challenging to predict parasite evolutionary responses without quantifying the extent of trait variation, including the extent to which parasites can adjust their traits in novel conditions. Assessing how fundamental variation in within-vector conditions affects parasites is necessary before it is possible to understand how complex human-induced factors like VCTs alter parasite ecology and evolution. Thus, I decided to focus on quantifying genetic variation for within-parasite traits, and examining how parasites and vectors respond to altered resource availability. I chose nutritional resource availability because all natural mosquito populations display individual variation in foraging success, perturbing nutrition is a simple manipulation in lab and semi-field systems, and previous studies observe changes in within-vector parasite development in response to additional blood meals (Shaw et al., 2020; Habtewold et al., 2021; Kwon et al., 2021). In addition, investigating altered resource availability is relevant to VCT use, because it can lead to shifts in foraging behaviours (Van Der Vinne et al., 2019), and is a consequence of the evolution of insecticide resistance mechanisms in mosquitoes. For example, insecticide resistant *Culex pipiens* mosquitoes had 30% less energetic reserves than their susceptible counterparts (Rivero et al., 2011), and insecticide resistant *An. coluzzii* have an increased metabolic rate (Ingham et al., 2021b).

7.2 Genetic variation for within-vector traits

In Chapter 3, I used three genetically distinct *P. chabaudi* genotypes to quantify differences between within-vector traits and onward transmission. I found that key transmission traits, including oocyst densities, sporozoite densities and the development time (extrinsic incubation period, EIP) differed across genotypes. Interestingly, I also discovered that there are genotype-specific differences in asexual replication after transmission from vector to host. Specifically, parasite genotype AS performed the best following transmission to the next host, despite performing very similarly to genotype AJ within the vector. In addition, I found that mice were exposed to 8-fold more sporozoites when bitten by mosquitoes infected with genotype ER, but this did not give ER an advantage over AS or AJ in the next host. This study is the first to explore intraspecific variation in within-vector traits and during onward

transmission, and provides preliminary evidence for genetic variation in within-vector parasite traits. In the context of parasite evolution, genetic variation is important because if a parasite trait is impacted by the consequences of VCTs, their potential to evolve in response is higher if pre-existing (standing) genetic variation is exposed (Barrett and Schluter, 2008) (see Figure 2.I, Chapter 2). For example, variation in EIP suggests that parasite genotypes with faster EIPs can be favoured in mosquito populations with reduced lifespans caused by insecticide resistance mechanisms and/or insecticide exposure (Viana et al., 2016; Brown et al., 2020).

Future work is needed to examine a wider bank of genotypes to better quantify the extent of genetic variation for within-vector traits and explore its drivers. Such drivers could include trade-offs and correlations between life history traits, such as quality versus quantity of sporozoites, because these could further explain the differences observed. For example, if fast development and high parasite burden within the vector are correlated, this ultimately leads to lower quality sporozoites because of the limited resources available for a large number of fast developing parasites. To be able to accurately assess trade-offs between traits, future studies should investigate whether genotypes differ in sporozoite expulsion (Oke et al., 2023), sporozoite quality (Beyer et al., 2021) and replication in the liver phase. Studies which examine parasite genotype differences in sporozoite infectivity *in vivo* (Aleshnick et al., 2020) and *in vitro* (Costa et al., 2018; Werling et al., 2019) could further elucidate which parasite traits have genetic variation available for selection to act upon. In addition, identifying within-vector and within-host trait correlations (e.g. are parasites virulent to the vertebrate host also fast growing in vectors?) will strengthen predictions for how changes to mosquito populations could alter clinical disease and epidemiology.

7.3 Phenotypic plasticity of parasite and vector traits

My findings in Chapters 4 and 5 reveal that both parasites and vectors exhibit phenotypic plasticity in response to the availability of nutritional resources to the mosquito. Specifically, in Chapter 4, I demonstrate that parasites modulate their growth in response to a change in resource availability in manners that appear to maximise fitness. For example, I find that parasites in low-resourced mosquitoes experienced a reduction in oocyst burden over time, but increased their late-stage oocyst growth more than parasites in more well-resourced mosquitoes, ensuring that overall parasite biomass was maintained. It remains challenging to explicitly test for whether the observed developmental patterns are adaptive and/or the result of

different resource constraints, because parasite phenotypes likely reflect a mixture of both (Schneider and Reece, 2021). For example, the reduction in oocyst burden could be the result of an adaptive apoptosis-like parasite strategy (Reece et al., 2011), constraints imposed by the mosquito immune system (Smith and Barillas-Mury, 2016), and/or simply limitation causing parasite starvation.

By connecting the sequential parasite traits expressed during transmission, theoretical modelling based on evolutionary ecology could predict the strategies that are adaptive to parasites experiencing different within-vector conditions, which could then be compared with patterns observed in natural populations (Birget et al., 2018; Childs and Prosper, 2020). Furthermore, examining different phenotypes can inform research to investigate underpinning causal mechanisms and potential cues that parasites could be using to sense changes in their environment. Single cell RNA sequencing technologies have recently been applied to within-vector life stages (Howick et al., 2019; Real et al., 2021; Witmer et al., 2021), and are valuable tools for suggesting underlying mechanisms. In addition, experimental perturbations that induce a parasite response in the absence of environmental change (i.e. conditionally controlling mechanistic gene expression or tricking parasites by providing different concentrations of cues in controlled, common garden, conditions (Carter et al., 2014)) could provide insight into the extent that parasites can adjust their phenotypes and the resulting fitness consequences. For example, testing how parasites respond to a wider range of sugar availability and tracking sporozoite expulsion (Oke et al., 2023) and/or sporozoite prevalence in the salivary glands over time could elucidate if parasites are making adaptive decisions or are constrained by resource availability. Taken together, my findings of Chapters 3 and 4 demonstrate that genotypic differences occur under common garden conditions, and parasite genotypes can plastically respond to variation in resource availability. Thus, my work has also provided the foundations for future studies to examine the extent of GxE in traits, which is important for parasite evolution because this could expose cryptic genetic variation to selection in novel environments, like mosquitoes altered by VCT use (Paaby and Rockman, 2014). It would be surprising if GxE did not exist for within-vector traits, given that it is observed for a variety of within-host traits (Reece et al., 2008; Pollitt et al., 2011b; Birget et al., 2019; Schneider and Reece, 2021).

In Chapter 5, I used a semi-field system to identify mosquito nutritional status as a driver for temporal shifts in vector foraging rhythms. While biting time of day has a genetic component, the majority of variation is expected to be driven by plasticity in response to the environment (Govella et al., 2023). My findings provide the first

evidence of an environmental driver for the biting time of day variation observed in the field, and demonstrates that vector foraging rhythms are indeed plastic. Thus, this chapter highlights the extent of within-vector environmental variation that parasites can be exposed to, not just in terms of resource availability, but also because taking a blood meal at the wrong time of day could lead to parasite development being misaligned with other rhythmic mosquito processes, including immune rhythms (Rund et al., 2011). This is important because vector foraging outside of the 'classical' biting window is likely to drive residual transmission in areas with high VCT coverage (Sherrard-Smith et al., 2019). My findings also establish the importance of considering how altered resource allocation (and other environmental changes) can impact on multiple parameters of parasite fitness, because they are affected in opposing ways. For example, sporozoite densities are reduced in low-resourced mosquitoes, but my findings in Chapter 5 suggest that low-resourced mosquitoes are more likely to host seek at unusual times outside of the 'classical' biting time window. Thus, investigating how both vector and parasite phenotypes respond to environmental change is necessary for understanding how natural selection will shape disease transmission and parasite evolution. Predicting the overarching impact of a specific environmental change on parasite fitness (i.e. R_0) requires mathematical modelling approaches, but empirical data like those collected in my thesis (i.e. the impact on both vector and parasite) is needed to parameterise these models (see Box 2.2, Chapter 2).

7.4 Novel methods for investigating within-vector parasite fitness

Estimating the impact of environmental change on within-vector parasite fitness is challenging, partly because tools that track parasite development over time in individual mosquitoes are lacking. While the extrinsic incubation period (EIP) of parasites has historically been estimated by the prevalence of sporozoites in the salivary glands (Ohm et al., 2018), this may not be the most appropriate measure of mosquito infectivity to vertebrate hosts. In addition, it is difficult to accurately assess the impact of alterations to the life history traits of individual mosquitoes on parasite development and onward transmission because current methods are destructive. While population-level measures are useful for modelling purposes (Ohm et al., 2018), tracking individual mosquitoes over time provides better resolution for the duration of parasite development and sporozoite production, allowing for better estimation of transmission potential throughout a mosquito's lifespan and in response to environmental variation (Guissou et al., 2021; Oke et al., 2023). Specifically, investigating when sporozoites are expelled from the glands is an important measure

for parasite fitness, because there is evidence that sporozoites may need to mature in the glands prior to transmission, and expelled sporozoites are transcriptionally different to those in the salivary glands (Bogale et al., 2021) so are more likely to be infectious to a vertebrate host.

In Chapter 6, I developed and tested a novel non-destructive assay to track sporozoite expulsion over time from individual and small groups of mosquitoes. While my method could detect expelled sporozoites, further work is needed to increase the detection rate to maximise the utility of the assay for quantifying transmission dynamics. This chapter also revealed key open questions regarding the potential biological causes underlying the low detection rate of sporozoites. For example, whether sporozoite expulsion is sporadic (Guissou et al., 2021) or whether there is variation in the number of sporozoites expelled due to clumping (Frischknecht et al., 2004). Recent studies have demonstrated that the number of sporozoites expelled positively correlates with infectivity to a vertebrate host (Kanatani et al., 2024), but whether there are genotypic differences between sporozoite expulsion, or whether parasites exhibit plasticity in the number of sporozoites expelled in response to within-vector environmental conditions is unknown. For example, examining whether the sporozoite inoculation size/infectivity correlation exists under different within-vector conditions could provide insight into the infectivity of individual sporozoites, and whether parasites are adaptively adjusting their sporozoite quality and/or inoculum size to maximise onward transmission potential.

7.5 Translational application of my findings

7.5.1 Control interventions

While I mostly used a rodent malaria model throughout my thesis, I predict that my fundamental findings will be largely applicable to human malaria. This is because rodent malaria systems can provide crucial insights into conserved aspects of parasite biology, and the model system *P. chabaudi* shares many similar characteristics with the deadliest human malaria parasite, *P. falciparum* (Simwela and Waters, 2022). These species also exhibit similar responses to environmental change within the host and vector. For example, the conversion rate and sex ratio strategies that *P. chabaudi* deploy within their host are consistent with those observed for *P. falciparum* (Schneider and Reece, 2021). I also found that within the vector, *P. chabaudi* sped up its growth in response to an additional blood meal in a similar way to *P. falciparum*, unlike the rodent malaria *P. berghei* which did not increase oocyst growth rate (Kwon

et al., 2021). Finally, the aim of my thesis was to test for proof-of-concept for fundamental evolutionary predictions, which explain the life history strategies of organisms across diverse taxa (including parasites). Therefore, *P. falciparum* and other human malaria parasites are unlikely to be exceptions to these rules.

The knowledge that parasites exhibit genotypic differences and phenotypic plasticity in within-vector traits suggests that parasites have the evolutionary potential to respond to within-vector changes in ways that can maximise fitness. VCT use has led to altered mosquito genotypes and phenotypes, including altered resource allocation, shifts in biting time of day and different mosquito species becoming more dominant as vectors (Oke et al., 2022). Thus, investigating within-vector parasite strategies, and discovering their limits and constraints, could reveal novel targets for interventions, including the most evolutionarily constrained traits. For example, my findings suggest that mosquito biting time is plastic and can shift dependent on environmental conditions. However, if shifts predominantly cause biting in the morning, this may constrain transmission and parasite evolution because mating within the mosquito midgut cannot occur at higher temperatures (Suh et al., 2020). In contrast, in poorly-resourced mosquitoes which have shifted to biting in the evening, parasites might not experience any disruption to mating and then ensure onward transmission in resource limited conditions by adjusting oocyst growth accordingly. Thus, explaining variation in both parasite and vector traits, and how they interact, is required to predict parasite responses to VCT use and maximise the success of current and future interventions. In addition, experimental evolution studies, where parasites are serially passaged via different types of vector, would reveal how parasites evolve and provide potential genetic markers for field monitoring. However, these experiments are challenging and likely require 10-15 life cycles to identify evolutionary changes (Mackinnon and Read, 1999b; Barclay et al., 2012). Thus, knowing which parasite traits exhibit high levels of genetic variation and/or phenotypic plasticity can direct future studies to investigate the VCT-induced changes which are most likely to drive parasite evolution.

7.5.2 Evolutionary ecology of other parasite systems

While malaria parasites have predominantly been used to investigate parasite life history traits, evolutionary ecology frameworks can also be applied to strategies observed in other parasites. For example, laboratory lines of *Trypanosoma brucei* parasites rapidly reduce transmissible ‘stumpy’ formation when they are not transmitted through their tsetse fly vector, suggesting that these parasites also

experience a survival-reproduction trade-off (Pollitt et al., 2011a; Rico et al., 2013). Trypanosomes also exhibit plasticity in within-host growth rate in response to stress in manners that appear adaptive. For example, *T. cruzi* slows its proliferation in response to nutrient restriction, but can speed up growth again once the constraint is released, demonstrating that parasites are able to resume growth once conditions become more favourable (Dumoulin and Burleigh, 2018). However, like malaria parasites, the within-vector ecology of other parasite systems has been overlooked. Some limited evidence suggests that parasites possess strategies for maximising transmission from vector to host. For example, *Schistosoma* spp. cercariae emerge from their snail vector at different times of day, dependent on whether their next host is nocturnal or diurnal (Lu et al., 2009; Su et al., 2013). Similar proof-of-concept studies like those in this thesis are needed to investigate other within-vector strategies of other vector-borne parasites which cause significant morbidity worldwide (e.g. *Schistosoma* spp., *Leishmania* spp., *Trypanosoma* spp.) (World Health Organization, 2017). Improving vector control capacity is a key priority for the World Health Organisation for all vector-borne diseases (World Health Organization, 2017), and thus it is necessary to investigate the evolutionary potential of parasites within their vectors to be able to predict which interventions will be the most robust to parasite counter-evolution, and identify novel targets for interventions.

7.5.3 Testing evolutionary theory in parasites

Explaining variation in parasite traits is important in its own right because parasites cause significant morbidity and mortality worldwide in human and animal populations, but they are also a good testing ground for evolutionary theory. For example, measuring reproductive strategies is challenging in multicellular organisms because they are often confounded by numerous aspects of reproductive investment, like parental care, and age-related improvement due to experience (Clutton-Brock, 1984). Instead, parasites offer a more tractable model system to experimentally test evolutionary theory (Poulin, 2007). In particular, malaria parasites are an excellent model system because they have well-defined and quantifiable traits (Reece et al., 2008). More broadly, parasites have a multitude of lifestyles and life cycles, often including multiple vectors/hosts. Thus, demonstrating that evolutionary theory originally developed to explain the strategies of free living multicellular organisms can also provide insight into parasite strategies strengthens support for the power and generality of a Darwinian approach.

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