The Impact of Erythrocyte density on the Fitness of *Anopheles gambiae* mosquitoes and their Capacity to Transmit Human Malaria

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Submitted August 2012 to the University of Glasgow for the degree of Doctor of Philosophy

How did the rose

Ever open its heart

And give to this world

All its beauty?

It felt the encouragement of light

Against its being,

Otherwise,

We all remain

Too frightened.

Hafez (Persian poet)

We especially need imagination in science. It is not all mathematics, nor all logic, but it is somewhat beauty and poetry

(Dr. Maria Montessori)

It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is the most adaptable to change.

(Charles Darwin)

To write simply is as difficult as to be good

(William Somerset Maugham)

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Acknowledgements

I would like to express my highest respect and deepest gratitude to those who made this thesis possible: The light of my life and creator of my essence, my God who always held me and tell me don't lose your wings in this sky so fly, fly and fly.

This beautiful country that hosted me, an Overseas Student Research Scholarship (ORS) from the University of Glasgow, and a scholarship from the Women Graduates (F*f*WG) Foundation for funding my last year expenses.

My special thanks go to Dr. Lisa Ranford-Cartwright and Dr. Heather Ferguson, my supervisors, for taking the time to be there when I needed you. Thanks for helping to see me through so much and giving me the opportunity to study my desired subject. Many thanks for lending me your books, specially "statistics for terrified biologist", introducing me to statistical modelling, illuminating my deepest affection for analysing biological data and their possible statistical correlations, and for introducing me to tiny vital bubbles over the midgut of mosquitoes "oocysts" and "QPCR" which prepared for me the opportunity to watch the signal of life. Many thanks for your encouragement, supervision and profound enthusiasm, stimulating discussion, patience and reading of, and commenting on this thesis through my PhD, and for remaining optimistic even when I was feeling hopeless, encouraging and guiding me through the beauty of English literature and scientific writing, and patiently explaining the root and sense of each words that I used in my English writing by everyday newspapers and my beloved magazines which made my days. Any later mistakes which remain are my own responsibility alone and I will promise to do each day much better than the previous day in terms of my beloved English writing. I heartily appreciate your kind financial support, during my writing up period. This acknowledgements would not be complete without mentioning all delicious treats such as gorgeous Sacher cakes from Wien, chocolates and biscuits from all around the world. Thank you for taking the moments out of your own precious days to make a little more sunlight shine in mine.

Sincere thanks to my assessors, Dr. Roman Biek and Prof. Daniel Haydon whose encouragement, advice and collaboration have helped me throughout my work, and for being a wonderful leader in the mountain, even though, I was not a good hiker.

Elizabeth Peat and Dorothy Armstrong for assistance with maintaining mosquitoes *An. gambiae* s.s colony, preparing the media required for malaria parasite culture, all the other valuable works and kind supports during the experiments, specially listening to my stories. Dr. Joseph Mugasa, for teaching me the tricks of PCR experiments, and cooking for me delicious African food. Dr. Jonathan Mwangi, for teaching me tissue culturing and logical calculation of parasites, and for his nice smile in necessary situations. Dr. Issa Lyimo, for teaching me how to work with mosquitoes in the insectary and transferring them so fast between little tubes, and for accompanying me in meetings. Dr. Neil Macritchie, for being a special soul and getting me a chance to discuss about human, wisdom and poems. Prof. Michael Barrett, for having nice discussions on English literature and different philosophers, specially my favourite writer and his book. Prof. Stephen Phillips for teaching me professional photography, and encouraging me by the sentence "Punctuality is pride of princess". Dr. Richard Reeve for pleasant informal statistical discussion on the whiteboards. Cory Meaden, for helping me in understanding the "feeling" of English language, and her companying me to the gym and lunch time chat. Lenka Richterova, for her company in hiking and photography, and preparing nice Easter eggs and cakes. Gordon Ramsay, for being the first happy face each morning, and for taking my "red cross Parcels" every month (my parent parcels), which always make me so happy. And my all lab colleagues and friends past and present, especially Dr. Laetitia Lempereur, Dr. Federica Giordani, Dr. Dong-Hyum Kim, Dr. Marco Morelli, Abhinav Sinha, Eduard Kerkhoven, David Wildridge, Katharina Johnston, Abdulsalam Alkhalidi, Sultan Al-ghamdi, Mikhail Churakov, Minnie Parmiter, Joaquin Prada, Narges Nafisi, Negar Ahani and Glayol Ebrahimi for their kind help and friendship.

At the end, I owe a very special thanks to people who make the world a better essence for me: my beloved parents (Seyedeh Fereshte Mousavi Algharavi, Seyed Mahmoud Emami), for teaching me to be brave and self dependent; illuminating the flame of discovery in my soul, for their love, trust and support during my study, and all of their efforts through my life. And also my brother and his family (Dr. Seyed Mohammad Amin Emami, Layla Abab and Seyed Daniel Emami), for encouraging me to be adventurous and enthusiasm in science. You are appreciated, more than words can express.

Declaration

I hereby solemnly declare that the research described within these pages is my own work except where stated, and truthfully acknowledge the contributions of all those collectively remembered, forgotten, and unrecognised, who went before.

Signed:

August 2012

Abstract

Anaemia is a common health problem affecting women and children in the developing world. This condition is characterized by a reduction in erythrocyte density, primarily resulting from malnutrition and/or infectious diseases such as malaria. As red blood cells are the most important source of protein for haematophagous mosquitoes, any reduction could impede the ability of mosquito vectors to transmit malaria by influencing their fitness and/or that of the parasites they transmit.

The aim of this study was to determine the impact of differences in the density of red blood cells in human blood on human malaria transmission (*P. falciparum*) by influencing: (i) mosquito vector (*An. gambiae* s.s.) fitness, (ii) the infectiousness of parasites to mosquitoes, (iii) parasite sporogonic success in mosquitoes, and/or (iv) the virulence of malaria parasites to mosquito vectors. The hypotheses tested are based on the premise that mosquito vector survival, fecundity and malaria parasite sporogony may be influenced by the nutritional value of infectious blood meals.

Mosquitoes (*An. gambiae*) were offered blood meals at different Packed Cell Volume (PCV) of human blood consistent with those arising from severe anaemia (low PCV= 15%) and normal PCV (50%), and containing malaria parasite (*P. falciparum*) gametocytes infectious to mosquitoes. Mosquito fitness, the success of parasite development within them, and/or the virulence mosquitoes experience from infection were evaluated.

The amount of protein that malaria vectors acquired from blood-feeding, and their resultant long term survival, was found to be positively associated with the PCV of human blood. Mosquitoes feeding on blood with low PCV had the same oviposition rates as those feeding on blood of normal PCV, but also showed an increase in fecundity of around 15%. Moreover, it was found that *An. gambiae* s.s. mosquitoes were substantially more likely to become infected after feeding on gametocyte-infected blood of low PCV rather than normal PCV. However, there was no evidence that blood PCV influenced either the oocyst intensity in infected mosquitoes, or the total number of transmission-stage sporozoites they developed by 10 days after the infectious blood meal. Finally, there was no evidence that either consumption of *P. falciparum*-infected blood or the subsequent development of oocysts reduced any measure of mosquito fitness

(oviposition, fecundity, survival), in either mosquitoes infected from blood of low or normal PCV. The impact of blood PCV on the energetic reserves of both infected and uninfected mosquitoes was found to be relatively minor.

These results demonstrate that variation in human erythrocyte of a magnitude likely to arise in malaria-endemic settings may have a significant impact of the outcome of vector –parasite interactions. Conditions such as severe anaemia, which reduce red blood density, could enhance malaria parasite transmission by increasing parasite infectivity to mosquitoes, while having no deleterious effects on mosquito survival.

Chapter 1. General Introduction

1.1 Background

Malaria is the most important vector-borne disease in the world. In 2010, it was estimated that approximately 1.13 to 1.44 billion people worldwide were exposed to the threat of malaria, with 225 million (range 146-316) clinical episodes attributable to this disease recorded (Snow *et al.*, 2005; Cibulskis *et al.*, 2011; Gething *et al.*, 2011). The risk of death from malaria is much higher in Africa (176 million) than in south east Asia, the western Pacific (49 million) or elsewhere (Snow *et al.*, 2005; Cibulskis *et al.*, 2011; Gething *et al.*, 2011). Although this disease is still one of the most important health problems in the world, especially in children (Talman *et al.*, 2004; Smith *et al.*, 2005), malaria-associated mortality and morbidity is dropping substantially in some areas (O'Meara *et al.*, 2008; Cibulskis *et al.*, 2011). This progress has been attributed to new vector control tools, the wider implementation of already-effective control approaches and increased investment into surveillance, control and treatment (O'Meara *et al.*, 2010).

Malaria transmission is determined by the outcome of interactions between *Plasmodium* parasites and their mosquito vectors. There has been much focus on the respective roles of these partners in determining the outcome of these interactions including the influence of parasite and mosquito genetics, and environment factors (Beier, 1998; Yan *et al.*, 1997; Lambrechts *et al.*, 2006; Mendes *et al.*, 2008; Niare *et al.*, 2002; Riehle *et al.*, 2006; Gouagna *et al.*, 2004a; Noden *et al.*, 1995; Okech *et al.*, 2004b; Mitri *et al.*, 2009; Drakeley *et al.*, 1999; Ferguson & Read, 2002a; Rogers & Randolph, 2000; Tripet *et al.*, 2008; Pascual *et al.*, 2006; Gilioli & Mariani, 2011; Paaijmans *et al.*, 2010; Dimopoulos *et al.*, 2002; Dong *et al.*, 2006; Baton *et al.*, 2009). Less attention has been paid to the influence of the vertebrate host condition on the interaction between parasites and vectors, and specifically how human host factors impact malaria transmission success in general. Currently, little is known about the role of human haematological factors on malaria transmission, either by influencing the infectiousness of parasites to mosquito vectors, and/or the fitness and transmission potential of mosquito vectors. Several human host haematological factors are influenced by malaria infection (Trampuz *et al.*, 2003; Meera *et al.*, 1999; Griffiths *et al.*, 2001; Thuma *et al.*, 2011), with one of the most notable being a reduction in the density of red blood cells due to anaemia (Akinboro *et*

al., 2010; Menendez *et al.*, 2000; Shulman *et al.*, 1996). As red blood cells are an important source of nutrition for both malaria parasites and their mosquito vectors, it is possible that variation in human red cell density could influence the ability of mosquitoes to carry parasites, and parasite developmental success.

The aim of this study is to determine whether variation in the red cell density of human blood influences the outcome of interactions between *Plasmodium* parasites and their mosquito vectors, and the efficiency of malaria transmission. Specifically, variation in the red blood cell density consistent with what would be expected in normal and severely anaemic humans was investigated. To address this issue, a series of experimental infection experiments were conducted in which the major mosquito vector of malaria in Africa, *An. gambiae s.s.*, were fed human blood infected with the transmission-stage gametocytes of *Plasmodium falciparum*, the most important malaria parasite of humans in Africa. The red blood cell density (as measured by the standard proxy measurement of Packed Cell Volume, PCV) of the infectious blood meals was varied to mimic that of normal and severely anaemic hosts.

A general overview of the malaria transmission system is now given to provide background on the potential importance and relevance of host blood PCV to malaria transmission, and the specific hypotheses that will be investigated.

1.2 Malaria

1.2.1 Parasite life cycle

Malaria is caused by parasites of the genus *Plasmodium*. Six species of these protozoa are currently known to be capable of infecting humans: *P. falciparum*, *P. vivax*, *P. ovale curtisi*, *P. ovale wallikeri, P. malariae* and *P. knowlesi* (Cox-Singh & Singh, 2008; Sutherland *et al.*, 2010). All malaria parasites require two hosts – the vertebrate host where asexual replication (schizogony) occurs, and the mosquito vector where sexual replication (sporogony occurs) (Figure 1.1).

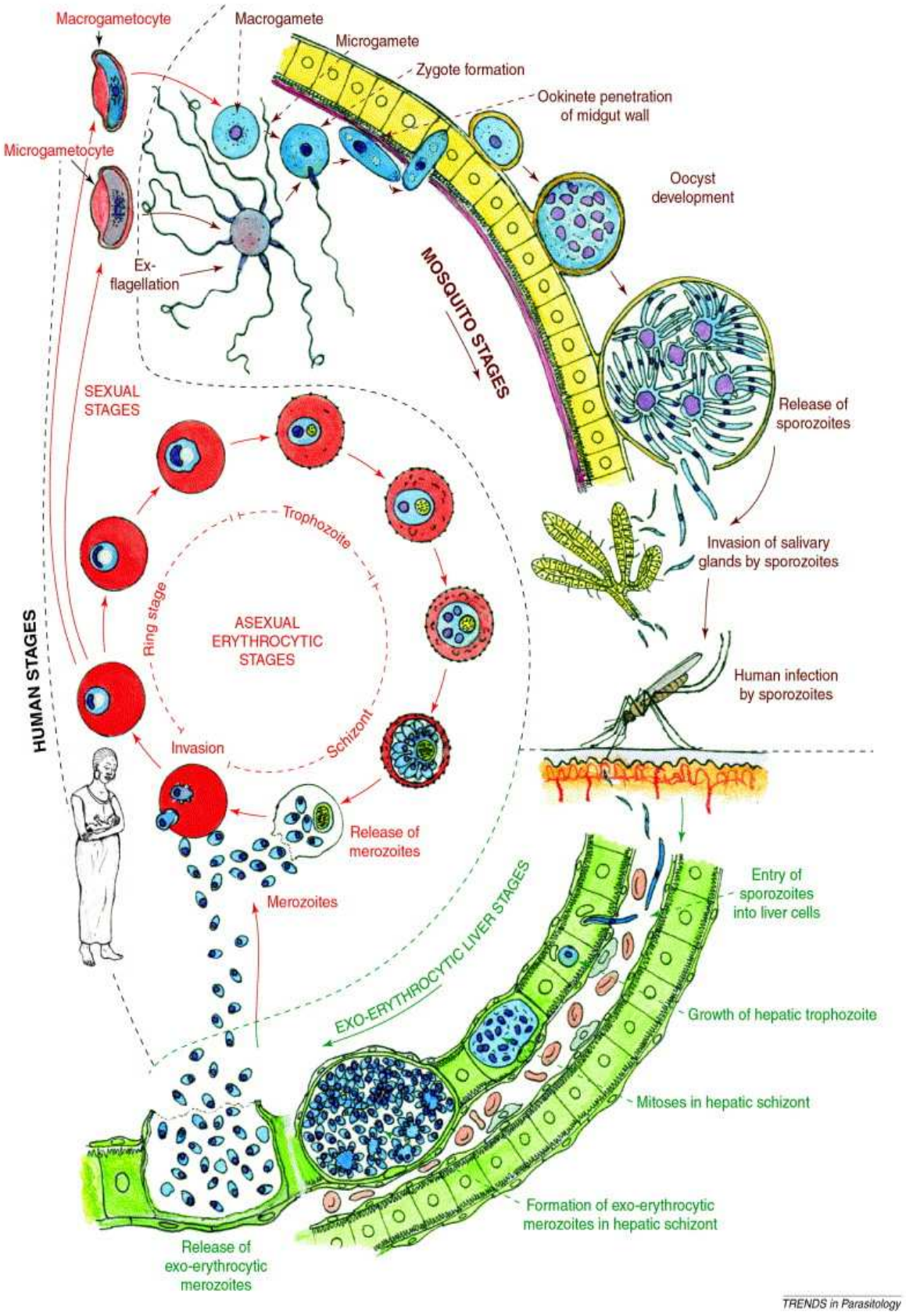

Figure 1.1. Malaria parasite (*P. falciparum*) life cycle [reproduced with permission from (Bannister & Mitchell, 2003)].

In the vertebrate host, an infection is initiated when sporozoites are injected with the saliva of a feeding mosquito (Ross & Thomson, 1910; Ross, 1898). Sporozoites are injected into dermis and move through it until they reach either blood (~70%) or lymphatic vessels (~30%) (Prudencio *et al.*, 2006). From there (within a few hours) most of the inoculated sporozoites are carried by the circulatory system to the liver where they invade hepatocytes and form a parasitophorous vacuole (Prudencio *et al.*, 2006; Amino *et al.*, 2006; Mota *et al.*, 2001). In each vacuole, *Plasmodium* sporozoites develop, multiply and generate thousands of merozoites (Prudencio *et al.*, 2006). This exoerythrocytic schizogony can generate 10,000-30,000 merozoites in 2-16 days, depending on the parasite species, which are then released into the bloodstream (Prudencio *et al.*, 2006; Janse & Waters, 2004a). In the bloodstream, merozoites invade erythrocytes where they undergo asexual replication. During this trophic period the parasite ingests host cell cytoplasm and breaks down ingested haemoglobin into amino acids (Bannister *et al.*, 2000). Nuclear division marks the end of the trophozoite stage and the beginning of the schizont stage. In tertian parasites like *Plasmodium falciparum*, the process from invasion of the uninfected erythrocyte to mature schizont takes around 48 hours, whereas in the quartan malaria (*P. malariae*) it takes 72 hours (Golgi, 1889). Whilst the majority of merozoites invading erythrocytes continue through the asexual cycle, a small fraction develop into sexual stage gametocytes which can be transmitted to mosquito vectors (Golgi, 1889; Carter & Miller, 1979). The presence of gametocytes in the peripheral blood generally arises 9-12 days after the initial asexual wave (Carter & Miller, 1979). All the merozoites within a particular schizont are destined to become either gametocytes or to continue the asexual cycle (Bruce *et al.*, 1990).

Ingestion of gametocytes by the mosquito vector induces gametogenesis (i.e., the production of gametes). Gametocyte maturation into gametes is triggered by changes in the environment such as a drop in temperature (which can be as much as 12^{o}C), an increase in pH, and exposure to xanthurenic acid (XA) (Sinden, 1983; Sinden *et al.*, 1996; Billker *et al.*, 1998), changes that occur in moving from the vertebrate to mosquito host. During this process, macrogametocytes (females) emerge from erythrocytes as non-motile macrogametes (Sinden, 1983). Microgametocytes (male) undergo a transformation termed exflagellation to form microgametes which emerge from erythrocytes (up to eight haploid microgametes per microgametocyte (Baton & Ranford-Cartwright, 2005a; Vlachou *et al.*, 2006; Baton & Ranford-Cartwright, 2005b). The motile

microgametes move through the blood meal until they reach a female gamete, when they fuse to form a diploid zygote. Over the next 18-24 hours the zygote transforms into a mature, motile ookinete, which crosses the mosquito peritrophic matrix and invades the midgut epithelial cells (Baton & Ranford-Cartwright, 2005b). Parasites come to rest on the basal lamina, about 24-48 h after the infective blood meal (Beier, 1998). From there, ookinetes transform into oocysts approximately 5-7 days after the infected blood meal. The developing oocyst undergoes multiple rounds of mitosis, which is marked by the appearance of one or more spindles in each nuclear lobe (Sinden & Strong, 1978a).

After about 10-14 days, oocyst development is completed and each oocyst will usually contain several hundred to thousands of sporozoites (Baton & Ranford-Cartwright, 2005b). Once mature the sporozoites are released passively into the mosquito haemolymph as a result of breakdown of the oocyst wall (Chen *et al.*, 1984). Sporozoites make their way from the haemolymph to the mosquito salivary glands (Pimenta *et al.*, 1994) from where they are ready to initiate a new infection when the mosquito bites (Meis *et al.*, 1992; Pimenta *et al.*, 1994; Akaki & Dvorak, 2005; Matuschewski, 2006). The total time required for complete parasite development in the mosquito ranges from 8 days upwards (Beier, 1998) and is highly temperature- and mosquito species- dependent; for example, *P. falciparum* takes 9 days at 30°C, 10 days at 25°C, 11 days at 24°C and more than 30 days at temperatures lower than 20 °C (Beier, 1998).

1.2.2 Mosquito vector life cycle

Mosquitoes belonging to the genus *Anopheles* and *Aedes* transmit human, bird and rodent malaria, but human malaria is only transmitted by female *Anopheles* mosquitoes (Grassi *et al.*, 1899). Of 476 *Anopheles* species that have been identified, only about 70 species are considered to transmit malaria (WHO, 2007; Service, 2012). Female mosquitoes are exposed to parasites when they feed on blood, a resource they require to provide energy and protein for the maturation of their eggs. In natural environments, *Anopheles* females seek a blood meal once every 2-4 days (Gillies, 1953), but they may feed daily or even several times a day if they are disturbed during their feeding (Beier, 1996). The eggs of *Anopheles* mosquitoes are deposited in or near water, in strings of 100-200 (Bruce-Chwatt, 1985). The length of time between a female mosquito taking a blood meal and laying her eggs is known as the gonotrophic cycle. Within an hour of

completing one gonotrophic cycle a female may start another (Clements, 2000). Through the mosquito's life the gonotrophic cycle is repeated in three biological phases: the search for a host and blood-feeding, digestion of the blood and egg maturation, and the search for a suitable oviposition site and oviposition (Lardeux *et al.*, 2008).

Blood meal ingestion triggers the production of eggs (vitellogenesis) in *Anopheles* (Raikhel & Dhadialla, 1992). The sequence of ovarian development in *Anopheles* mosquitoes has been divided into five stages, namely Christophers stages (Christophers, 1911). Vitellogenesis starts approximately ten hours post blood feeding when most follicles (stage II) start to deposit yolk (Adham *et al.*, 2009). Vitellogenin and other yolk proteins are synthesised and secreted by the fat body, and then transported to the ovary for incorporation into the developing oocytes (Lea *et al.*, 1978; Hagedorn, 1989). Mosquito eggs are matured continuously in batches, following periodic blood meal feeding (Clements, 2000).

Larval and adult mosquitoes live in different habitats (larvae are aquatic, adults are terrestrial), and feed on different resources. *Anopheles* mosquitoes lay their eggs on the surface of water, and the first instar larvae emerge within 1-2 days. The juvenile form of the insect passes through both larval and pupal stages. The growing mosquito larva moults 4 times before pupation. On the first three moults the larva does not change very much but during the fourth moult, it changes into a version of an adult and the organism that leaves the fourth larval skin is a pupa (Clements, 1992). When the adult is completely formed within the pupal cuticle, the mosquito rests at the water surface and starts to swallow air. The consequent increase in internal pressure causes a split along the midline of the pupal thoracic cuticle, and the adult slowly expands out of the pupal cuticle and steps onto the water surface [eclosion] (Clements, 2000). Larval development from egg to pupation is known to be influenced by numerous environmental factors such as temperature, larval density and nutrition (Huang *et al.*, 2006; Timmermann & Briegel, 1993; Thompson, 1976; Kaufman *et al.*, 2006; Koenraadt *et al.*, 2004; Kirby & Lindsay, 2009; Ranjeeta *et al.*, 2008). In natural populations, malaria vectors such as *An. arabiensis* and *An. gambiae* s.s are estimated to take 10 - 12 days to develop from egg to pupae (Beier *et al.*, 1990a).

In their aquatic habitats, mosquito larvae generally feed upon microorganisms whose availability has been linked to larval survival. Low food concentration in aquatic habitats reduces larval growth rates and the survival of *An. gambiae* larvae (Koenraadt *et al.*, 2004). Low food abundance in larval habitats may influence the population densities of adult mosquitoes such as *An. gambiae* and *An. arabiensis* because under starvation conditions, first-instar larvae can be consumed by fourth-instar larvae of the same species [cannibalism] (Koenraadt & Takken, 2003; Koenraadt *et al.*, 2004). The composition of food in larval habitats, as well as its quantity, can also influence mosquito development and adult survival (Gomulski, 1985; Suwanchaichinda & Paskewitz, 1998; Mercer, 1999; Reiskind & Lounibos, 2009; Kirby & Lindsay, 2009; Aboagye-Antwi & Tripet, 2010). Thus the composition and abundance of resources are key determinants of adult emergence rates and fitness.

On emergence from larval habitats, both female and male adult mosquitoes can feed on plant juices as a source of sugar. Usually this is obtained from plant nectaries, but sometimes also from other sources such as rotting fruit, honeydew and from damaged and intact vegetative tissues (Clements, 2000). Amino acids such as proline, arginine, alanine, serine and threonine are also present in the nectar of most plants (Baker & Baker, 1973; Baker *et al.*, 1983) and in aphid honeydew (Auclair 1963) but their concentration is insufficient for mosquito ovarian development. Female mosquitoes must find an alternative protein source (Auclair 1963). For most mosquitoes, these resources are primarily obtained from vertebrate blood (Clements, 2000).

To acquire a blood meal, female mosquitoes must first locate hosts using a variety of odour, carbon dioxide and temperature cues which are known to stimulate sense receptors on their antennae and palps (Clements, 2000). Female mosquitoes show a degree of specificity in their choice of host, whether mammal, bird or cold-blooded vertebrates, with some species being extremely specific in their choice of host species (Lyimo & Ferguson, 2009). Most *Anopheles* mosquitoes blood feed in the early evening, at night or in the early morning (Clements, 2000; Service, 2012). The location of biting often varies in a species-specific manner. For example, some malaria vectors bite humans mainly outdoors (exophagic), whereas others (e.g. *An. gambiae s.s.*) bite mostly indoors (endophagic) (Service, 2012).

In order to take a blood meal, the mosquito's probing stylets pierce a blood vessel and saliva is injected. If the female is undisturbed, blood feeding continues until abdominal stretch receptors send a signal of repletion (Clements, 2000). Within a few minutes a gorging mosquito can imbibe up to four times her own weight of blood (Clements, 1992). This provides the protein needed for egg production but also has some detrimental impacts such as the intake of a large water load which can make flight difficult, and toxic amounts of sodium, potassium and haem that are present in blood. To overcome the weight problem, some species of mosquito, including *An. gambiae* and *An. arabiensis*, discharge small volumes of fluid during feeding, a process termed pre-diuresis (Clements, 1992). Further water and salt is removed from the blood meal by diuresis in the malphigian tubules. As the mosquito feeds, the peritrophic membrane forms around the blood meal, separating it from the midgut epithelial cells (Billingsley & Rudin, 1992). Digestive enzymes such as trypsin and aminopeptidases are synthesised in the midgut epithelial cells after the blood meal, and then secreted into the midgut lumen (Billingsley & Hecker, 1991; Borovsky, 2003). By 24h after blood feeding, most of the proteins from the blood meal have been digested (Graf *et al.*, 1986). When digestion is completed the peritrophic membrane is discharged with haematin and other residues of the blood meal (Graf *et al.*, 1986; Clements, 2000). After feeding, *Anopheles* species require a resting period of 2-3 days for egg maturation (Clements, 2000). Many African malaria vectors rest predominantly inside houses or closed living shelters (endophilic), whereas the other species may use outdoor (exophilic) resting sites such as rodent burrows, in caves or cracks in the ground, in termite mounds or vegetation.

Mating usually occurs prior to blood feeding in mosquitoes. Only one mating is required for female mosquitoes (Clements, 2000), and sperm is then stored in the spermatheca to fertilise all subsequent eggs laid during their life time (Clements, 2000). Mosquito mating happens shortly after emergence of the adult from the pupa.

1.2.3 Malaria symptoms

A patient with *P. falciparum* malaria can present with a range of clinical severity from asymptomatic malaria, mild disease, or severe malaria (Sherman & Sherman, 1998). Mild malaria is frequently reported to be associated with mild to moderate anaemia (5- 10.9 < 11 g/dl) and fever symptoms (Obonyo *et al.*, 2007). Severe malaria symptoms include

cerebral malaria (Lopansri *et al.*, 2003; Grau *et al.*, 2003; Grau & Craig, 2012), pulmonary oedema, respiratory distress (Lackritz *et al.*, 1992), acute renal failure (Trang *et al.*, 1992), severe anaemia (Hb < 5 g/dl) (Bouyou-Akotet *et al.*, 2009), acidosis and hypoglycaemia (Elased & Playfair, 1994), and coma (Trampuz *et al.*, 2003; Nacher *et al.*, 2002). These complications can progress quickly and result in death within hours or days (WHO, 2011b).

Anaemia, characterized by a reduction in red cell density, is a common pathology associated with malaria infection, especially in *P. falciparum* (Trampuz et al., 2003). This condition is often viewed as an unavoidable consequence of the schizogonic cycle in which parasites rupture erythrocytes, and trigger further extensive lysis of uninfected cells and their removal by the patient's spleen (Bouyou-Akotet *et al.*, 2009; Buffet *et al.*, 2011; Awandare *et al.*, 2011). Clinical anaemia is defined by the magnitude of reduction in blood haematocrit (as measured by Packed cell volume, PCV) and/or concentration of haemoglobin (Beaton, 2000; Fairbanks & Tefferi, 2000). Packed cell volume and haemoglobin measurements are considered to be haematological indicators for classifying the severity of anaemia, with PCVs in the range of 21-30% and Hb 9.5- 13 g/dl being considered as mild anaemia, PCVs of 15- 20% and Hb 8- 9.5 g/dl as moderate anaemia, and < 15% PCV and Hb < 8 g/dl as severe anaemia (Mukiibi *et al.*, 1995). The normal range of PCV is 40- 50% (Hb 14- 18 g/dl) for adult African males, and 30- 46%, (13- 15 g/dl Hb) for adult females respectively (Fairbanks & Tefferi, 2000). Haematological variation in red cell density across this spectrum of disease severity could significantly influence the abundance and composition of blood energetic resources available for uptake by malaria parasites and their mosquito vectors, and thus transmission potential.

1.2.4 Malaria epidemiology

The geographical distribution of malaria (Figure 1.2) generally corresponds to areas of the world with climates suitable for transmission (Snow *et al.*, 2005; Gething *et al.*, 2011).

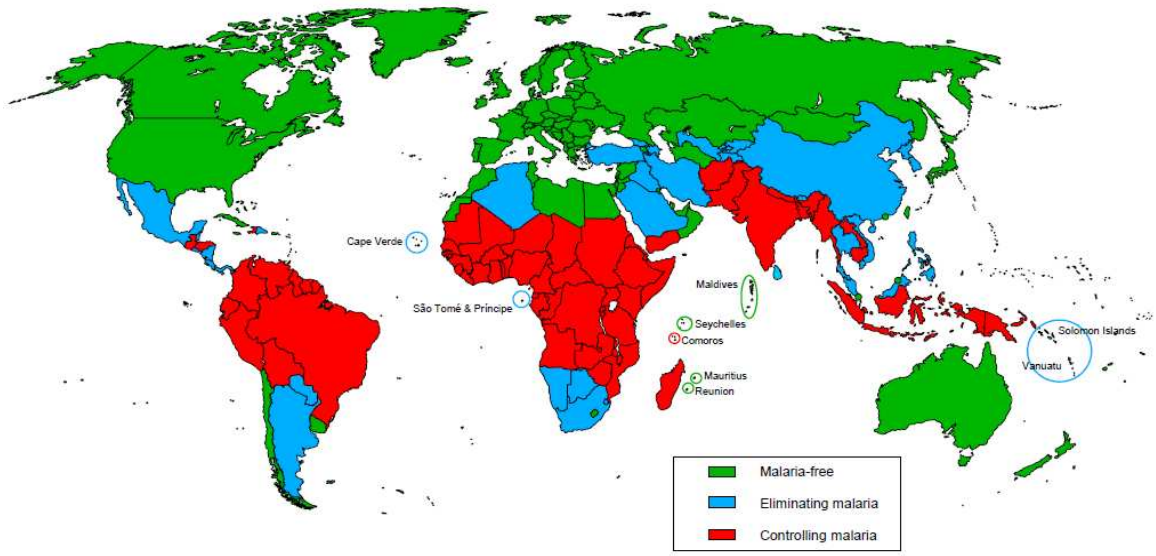

Figure 1.2. Malaria elimination initiative (2011), the global health group, University of California [taken with permission from (Cotter *et al.*, 2011)].

Mathematical models have played an important role in helping to explain and predict the epidemiology of malaria. The earliest attempt to provide a quantitative understanding of the dynamics of malaria transmission was by Ross (1911). His model included the concept of a threshold density of mosquitoes for malaria transmission, and demonstrated that in order to eliminate malaria, it was not necessary to eliminate *Anopheles* entirely, but only to reduce their number below a certain figure. The basic reproduction number (R_o) as a means to characterize malaria transmission was first introduced by Macdonald (1956). This parameter is defined as the average number of secondary infections produced by a primary infection. Here the parameter *r* is the rate of recovery in humans, such that *1/r* is the duration of infection in humans, *g* is the *per capita* daily death rate of mosquito vectors such that *1/g* is the average lifespan of a mosquito, *m* is the number of mosquitoes per human host, *a* is the rate of biting on humans per mosquito per day, *b* is the probability that an uninfected human becomes infected after being bitten by an infectious mosquito, *c* is the transmission efficiency of parasites from a human to a mosquito vector, and *n* is the length of parasite extrinsic incubation period [time from ingestion of gametocytes in blood by the mosquito until it develops transmission stage sporozoites] (Macdonald, 1956a; Smith *et al.*, 2005; Smith *et al.*, 2007).

(Equation 1) $R_o = m\, a^2\, bce^{-gn} / rg$

Macdonald (1957) demonstrated that of all the parameters in this equation, transmission is most sensitive to variation in adult mosquito survival, followed by human biting rate. This led him to conclude that malaria control efforts should prioritize interventions that reduce adult mosquito survival (Macdonald, 1957), which led to DDT spraying being adopted as the primary vector control strategy in the last Global Malaria Eradication campaign of the 1950-1960s (Ruan *et al.*, 2008).

The R_o equation was further modified by Garrett-Jones (Garrett-Jones, 1964) to focus specifically on the entomological predictors of malaria transmission. This index is known as vectorial capacity and is defined similarly to the reproductive rate R_o but with all the human host parameters removed. Consequently vectorial capacity describes the transmission potential of a mosquito population (Smith *et al.*, 2005). This index is particularly useful for investigating the relative contribution of mosquito fitness and behaviour to malaria transmission, which is the prime focus of this thesis. Parameters in this equation are as defined for R_o, with p being the estimated daily survival of mosquitoes, and n the length of the extrinsic incubation period (sporogonic cycle) of the parasite (Garrett-Jones, 1964; Smith *et al.*, 2005).

(Equation 2) $C = ma^2p^n / - logp$

Both these transmission parameters indicate that the infectiousness of parasites to mosquitoes, parasite development within mosquitoes and mosquito survival combine to have a major impact on malaria transmission. Thus any factor that could simultaneously influence all of these traits, such as variation in human red blood cell density as investigated here, could scale-up to influence malaria transmission.

1.2.5 Malaria Control

The most effective and common strategies to control malaria are anti-malarial drugs to cure infected patients and insecticides to control mosquito vectors (Pates & Curtis, 2005; Butcher *et al.*, 2000; Skarbinski *et al.*, 2012; Service, 2012; Castelli *et al.*, 2010). At present there is no effective vaccine against malaria (Kappe & Mikolajczak, 2011; Good,

2011) although encouraging results have recently been reported from early-phase clinical trials of some candidates (Good, 2011; Kappe & Mikolajczak, 2011). Consequently at present, vector control is considered to be the primary intervention strategy for reducing malaria transmission on a large scale (WHO, 2011a).

Vector control can be aimed at either the larvae and/or adult stages. Adult mosquito control approaches are considered to be the most effective and are the most widely used, although substantial progress with larval control has been achieved in some settings (Fillinger *et al.*, 2008; Fillinger & Lindsay, 2011). Control methods directed at immature stages include the use of biological control agents (e.g. predators, pathogens in larval habitats), physical or environmental control (e.g. source reduction and drainage); chemical control (e.g. larvicides) and insect growth regulators [IGRs] (Lou & Zhao, 2011; Govindarajan *et al.*, 2012; Kinde-Gazard & Baglo, 2012; Kihampa *et al.*, 2009; Dehne, 1955; Abdul-Ghani *et al.*, 2012; Service, 2012). In addition, there are several methods directed at mosquito adult stages. The most widespread and successful of these methods are indoor residual spraying (IRS), and insecticide-treated nets (ITNs) (Sharma et al., 2006; Lindblade et al., 2006; Quinones et al., 1997; Skarbinski et al., 2012; Bonner et al., 2011; Butcher et al., 2000; Service, 2012). These methods work by putting mosquitoes in contact with insecticides when they try to bite indoors at night, or when they rest inside houses (Pates & Curtis, 2005; WHO, 1992). ITNs are typically impregnated with pyrethroid insecticides (permethrin, deltamethrin, alpha-cypermethrin and lambda-cyhalothrin) (Lindblade *et al.*, 2006; Coleman *et al.*, 2006), and can remain effective for around 6-12 months after treatment (Lindblade *et al.*, 2006). Recently, long-lasting insecticidal nets (LLINs) have been developed which show great success in controlling malaria vectors (Bonner *et al.*, 2011) because they can remain effective for up to five years (Sharma *et al.*, 2006). These nets have a mass killing effect on vector populations and are the frontline recommended vector control strategy by the WHO (Bonner *et al.*, 2011).

Indoor residual spraying (IRS) is the spraying of residual insecticides on the surfaces of walls, ceilings and roofs of houses (WHO, 1992). Dichloro-diphenyl-trichoroethane (DDT: organochlorine) has been one of the most effective insecticides used for this purpose, but it is not widely used in many countries because of its eco-toxicity and the development of resistance by mosquito vectors (WHO, 2009; USAID, 2009). Alternative classes of

insecticides such as organophosphates (malathion, fenitrothion or pirimiphos-methyl), carbamates (propoxur, bendiocarb) and pyrethroids (lambda-cyhalothrin, deltamethrin or permethrin) are also used for IRS (Rivero *et al.*, 2010; Coleman *et al.*, 2006).

These insecticide-based control strategies have had a great impact on reducing malaria transmission and are being rapidly scaled up across Africa. For example, the number of (LLINs) in malaria –endemic regions in sub-Saharan Africa increased from 88.5 million 2009 to 145 million in 2010 (WHO, 2011a). However, as of 2011, 45 countries around the world have been reported resistance to at least one of the four classes of insecticides used in these tools including 27 countries from sub-Saharan Africa (Cibulskis *et al.*, 2011). Consequently, insecticide resistance and environmental change may pose a threat to their future effectiveness (Robert, 2007; Service, 2012) and there is a need for new control methods to accomplish the major goal of controlling and eradicating malaria on a wider scale (Walther & Walther, 2007; Pates & Curtis, 2005; WHO, 2005; WHO, 2011a; Service, 2012). Understanding the role of host factors in promoting or limiting malaria transmission can enhance the search for better control measures by helping to improve predictions of malaria epidemiology and the stability of transmission.

1.3 General hypothesis

As demonstrated by the Ross-MacDonald model, the intensity of malaria transmission crucially depends on several entomological and parasite transmission traits. Prime amongst these is adult mosquito survival, as only anophelines surviving longer than the parasite's extrinsic incubation period can transmit malaria after taking an infective blood meal. Also crucial is the frequency with which mosquitoes blood feed (Beier, 1996), and the success of parasite transmission to mosquitoes and parasite development within the vector. Other mosquito fitness traits such as reproductive success may indirectly impact malaria transmission by influencing the growth rate of vector populations.

In this thesis, I will investigate the hypothesis that the density of red blood cells in human blood could have a significant impact on malaria transmission by influencing: (i) mosquito vector fitness, (ii) the infectiousness of parasites to mosquitoes, (iii) parasite sporogonic success in mosquitoes, and/or (iv) the virulence of malaria parasites to mosquito vectors. This hypothesis is based on the premise that mosquito vector survival, fecundity and

malaria parasite sporogony may be influenced by the nutritional value of infectious blood meals. As other aspects of mosquito nutrition including sugar feeding and blood biochemical composition are known to influence mosquito fitness and malaria parasite sporogonic success (Andersson, 1992; Bowen & Romo, 1995; Gray & Bradley, 2006; Lopez & Mathers, 2006; Manda *et al.*, 2007b; Tirados *et al.*, 2006; Taylor & Hurd, 2001; Hurd *et al.*, 1995; Shieh & Rossignol, 1992), it is reasonable to hypothesize that variation in other host haematological factors that influence the resource value of blood to mosquitoes could have an impact on vectorial capacity.

In this study, the prime haematological trait of interest is human host red cell density, as measured by Packed Cell Volume (PCV). In order to assess the potential for PCV variation to influence parasite transmission, it is useful to first review the known determinants of mosquito fitness and parasite sporogonic success and their dependence on other environmental and mosquito nutritional factors.

1.3.1 Determinants of malaria vector fitness

Fitness is a complex trait that can be defined as the relative ability of an individual to leave descendents in the same population. Fitness of individual genotype is usually influenced by the environment in which the individual lives and therefore is not fixed for a given genotype. Fitness is best measured by the change of frequency in an individual genotype through several generations within a population (Lambrechts *et al.*, 2008; Roff, 2008). While fitness is best estimated through such multi-generation monitoring, in the shorter-term it may be approximated from variation in the life-history and demographic traits that influence the potential of an individual to contribute to the next generation in terms of their lifetime reproductive success. In the context of this thesis, this measure is indirectly estimated on the basis of two traits that combine to determine mosquito lifetime reproductive success: fecundity and adult survival. Of those traits, survival also has an additional, direct impact on malaria transmission by influencing the probability that parasites can complete their extrinsic development before their vectors die. Mosquito fecundity may also be indirectly related to malaria transmission through its potential to influence mosquito population growth rate (Russell *et al.*, 2011); although no clear relationship between mosquito fecundity and malaria transmission has yet been

demonstrated. A variety of environmental and innate biological properties are known to influence these mosquito fitness traits under natural conditions as reviewed below.

1.3.1.1 Fecundity

One of the most important determinants of mosquito fecundity is their adult body size (Briegel, 1990b; Briegel, 1990a; Takken *et al.*, 1998). Larval food supply is the primary determinant of adult mosquito body size, and is also positively correlated with the total protein, lipid and carbohydrate reserves of mosquitoes at eclosion (Briegel, 1990b). Body size is also important because it determines mosquito blood meal size, which is directly and positively correlated with fecundity (Hurd *et al.*, 1995; Ichimori, 1989; Canyon *et al.*, 1999; Nayar & Sauerman, 1975; Andersson, 1992). Blood consumption by large females is more than twice that of small mosquitoes, with fecundity over the same range increasing about 4-fold (Briegel, 1990). The efficiency of blood meal utilization for yolk synthesis is thus correlated to body size (Briegel, 1990a).

Mosquito body size is also a critical determinant of whether egg production will be initiated after feeding. For example, females of *An. gambiae* failed to mature their eggs unless their wing length was greater than 2.8mm (Fernandes & Briegel, 2005). In general, the smaller the existing level of energetic reserves at mosquito eclosion (as determined by larval nutrition), the larger the blood meal required to initiate egg development (Romoser *et al.*, 1989; Klowden, 1986). Above a critical level of blood intake, egg production increases with the volume of blood ingested (Briegel & Rezzonico, 1985a).

Female reproductive success may be influenced by sugar-feeding in addition to blood feeding, however the effect of sugar availability on mosquito fecundity appears to vary between mosquito species and settings. For example, one study showed that the lifetime fecundity of *Ae. aegypti* mosquitoes was not significantly affected by sugar availability (Braks et al., 2006), while others reported that sugar feeding delayed oviposition in gravid females and thus reduced mosquito fecundity (Gary & Foster, 2006; Shroyer & Sanders, 1977). A potential negative impact of sugar feeding on mosquito fecundity is that it may reduce the desire of mosquitoes to blood feed (Foster & Eischen, 1987; Nayar & Sauerman, 1975). For example when deprived of sugar sources, *An. gambiae* mosquitoes $\geq$ 20 days old had a higher biting rate than those which had sugar available. However,

young (5-12 d) and intermediate aged (13-19 d) mosquitoes showed no differences in blood-feeding pattern in response to sugar availability (Straif & Beier, 1996). Sugar-feeding may also influence the 'quality' of eggs produced by mosquitoes. For example, eggs produced by sugar-deprived *Ae. taeniorhynchus* had lower lipid content than those provided with *ad libitum* access (Nayar & Sauerman, 1975). As egg lipid content is positively correlated with egg hatching rate, this effect could influence mosquito fitness (Timmermann & Briegel, 1996; Atella & Shahabuddin, 2002). However, so far sugar deprivation in *Anopheles* does not appear to affect the caloric content of eggs (Briegel, 1990b; Foster, 1995). In summary, sugar-feeding has been shown to have numerous impacts on mosquito fecundity, but the nature of these effects is variable and depends on the study system and aspect of reproduction under consideration.

In addition to larval resources and sugar feeding, the composition of vertebrate blood also has a significant impact on mosquito fecundity. For example, human blood has lower isoleucine content than rodent blood (Harrington *et al.*, 2001), and mosquitoes (*Ae. aegypti*) feeding on human blood frequently and lay more eggs than the same species feeding on rodent blood (Harrington *et al.*, 2001). Similar host-species specific impacts on mosquito fecundity have been demonstrated in other systems (Lyimo & Ferguson, 2009), but not in *An. arabiensis* or *An. gambiae* s.s. (Lyimo *et al.*, 2012). The direct influence of host haematological factors such as red blood cell density on mosquito fecundity has not been investigated.

1.3.1.2 Survival

As with fecundity, mosquito body size has a strong positive correlation with adult survival (Andersson, 1992; Lyimo & Takken, 1993; Gimnig *et al.*, 2002; Briegel, 1990a). As adult mosquito body size is set primarily by larval reserves (reviewed above), food availability in larval habitats is thus a prime determinant of adult survival (Lowrie, 1973; Kirby & Lindsay, 2009; Reiskind & Lounibos, 2009; Barrera & Medialdea, 1996). Another important nutritional resource for adult mosquito survival is sugars. Under laboratory conditions, female *Ae. aegypti*, *Cx. quinquefaciatus* and *An. gambiae* live longer when provided with both sugar and blood meals rather than blood meals alone (Harada *et al.*, 1976; Manda *et al.*, 2007b; Okech *et al.*, 2003). Consequently, the availability of sugar increases the survival potential of vectors beyond ages at which they are old enough to

transmit malaria (Andersson, 1992; Okech *et al.*, 2003; Manda *et al.*, 2007a), and sugar is thought to be a potentially crucial determinant of vectorial capacity (Manda *et al.*, 2007b).

Although primarily considered to be a critical resource for reproduction, vertebrate blood may also influence mosquito long-term survival. Laboratory studies have shown that resources from host blood can enhance mosquito survival (Briegel & Rezzonico, 1985a; Lea *et al.*, 1978; Briegel, 1990a; Hogg & Hurd, 1995a; Lyimo & Ferguson, 2009). The impact of blood on mosquito survival may vary between host species (Lyimo & Ferguson, 2009). For example, *Aedes aegypti* females fed on human blood had greater survival than those fed on non-human blood [rodent] (Harrington *et al.*, 2001), and *An. gambiae s*.s. fed on human or cow blood lived significantly longer than those fed chicken or dog blood (Lyimo et al 2012). A potential explanation for variation in the survival value of different blood types to mosquitoes is their impact on protein and lipid storage. The most important energetic reserve for mosquito survival is lipids (Vanhandel, 1984; Briegel *et al.*, 2001; Huho *et al.*, 2007; Arrese & Soulages, 2010), and this and other key resources including carbohydrates and protein are known to be positively correlated with adult survival in a range of mosquito taxa (Timmermann & Briegel, 1999). While most of these resources are acquired during larval development, further contributions may be made from blood imbibed at the adult stage. Very little is known, however, about how haematological variation within a host species influence the abundance of mosquito energetic reserves and their subsequent survival. Understanding this important gap in our knowledge is one of the key objectives of the current thesis.

1.3.1.3 Other indirect determinants of mosquito vector fitness

Other behavioural and physiological traits such as mosquito flight ability may indirectly influence mosquito fitness by determining their ability to locate hosts and breeding sites, and to avoid predators. Mosquito flight ability is known to be dependent on their nutrition. Adult mosquitoes with low carbohydrate reserves have limited flight ability (Andersson, 1992; Nayar & Vanhande, 1971; Kaufmann & Briegel, 2004). Mosquitoes can utilise blood nutrients obtained from feeding for energy for flight, as part of the digestion products are converted to glycogen and triacylglycerol (Kaufmann & Brown, 2008). Blood-fed *An. gambiae* can fly further than those fed on sugar alone (Kaufmann & Briegel,

2004). Host haematological variation such as PCV might also influence flight and other crucial behavioural activities (oviposition, evading predators) by altering the energy available for metabolism and activity.

1.3.2 Determinants of malaria parasite infectiousness to mosquitoes

Here infectiousness is defined as the probability that gametocytes within a blood meal will establish an infection in a mosquito, which is measured by the presence of oocysts within mosquitoes. Following this definition, the infectiousness of malaria parasites to vectors has been associated with a range of parasite, mosquito, host and environmental factors. In *Anopheles* mosquitoes, susceptibility to human malaria parasites varies between mosquito parasite species (Sherman & Sherman, 1998). Mosquito immunity is also a critical determinant of whether infection will occur. In laboratory settings, several mosquitoes have been shown to mount immune responses against parasites including the melanization of oocysts (Collins *et al.*, 1986; Paskewitz *et al.*, 1988) and ookinete lysis during passage through the gut epithelial cells (Vernick *et al.*, 1995). The ability of mosquitoes to mount a melanisation response can be affected by their nutritional state: *Anopheles gambiae* mosquitoes were more likely to melanise negatively charged Sephadex beads injected into the haemolymph after ingesting a blood meal compared to mosquitoes which were unfed (Schwartz & Koella, 2002). However melanisation is not believed to play a major role in controlling human *Plasmodium* infections in naturally infected mosquitoes (Riehle *et al.*, 2006); thus the relevance of these reported impacts of blood availability to mosquito immunity are unclear.

Ookinete lysis may be a more evident immune response in natural mosquito infections, but its cellular basis has not yet been characterised (Vernick *et al.*, 1995). Parasites can also be prevented from establishing a midgut oocyst infection by the death and ejection from the midgut of ookinete-invaded epithelial cells (Han *et al.*, 2000; Baton & Ranford-Cartwright, 2004). Nitric oxide (NO) is a molecule that is part of a peroxidase induction cascade that initiates apoptosis in mosquito midgut cells (Kumar, 2004). NO synthesis requires arginine, a key component of other physiological pathways such as egg production and sperm maturation in insects that can only be obtained through the insect diet (Tripet, 2008). Although it therefore seems likely that the ability to produce NO will

be correlated to insect diet, there is currently no evidence that host blood factors are correlated with such mosquito anti-parasitic responses under natural conditions.

Several other factors such as parasite genotype, environmental factors, parasite gametocyte density, blood digestion, variation in host PCV have been shown to influence parasite infectiousness to mosquitoes in a range of laboratory and field systems. It was shown that malaria infection rate to mosquitoes varied significantly between parasite genotypes in a rodent laboratory model (Ferguson *et al.*, 2003b), and is also influenced by parasite genetic factors in natural settings (Niare *et al.*, 2002). The number of oocysts that develop within a mosquito has also been associated with mosquito factors such as their body size (Lyimo & Koella, 1992). Environmental influences such as temperature have thresholds above and below which infections does not occur. The infection rate and oocyst load of *P. falciparum* in mosquitoes (*An. stephensi*) is not affected by to lower temperatures (21-27°C) or mosquito age (Noden *et al.*, 1995; Okech *et al.*, 2004b), although earlier studies reported that the lowest threshold temperature for *P. falciparum* development is at 16°C (Detinova, 1962). High environmental temperatures (30 and 32°C) limit the parasite infection rate and oocyst load (Noden *et al.*, 1995). Other extrinsic factors such as the abundance of microbiota in larval habitats have been associated with oocyst intensities in *An. gambiae* mosquitoes (Okech *et al.*, 2007). The availability of blood resources both before and after infection may also influence the infectiousness of malara parasites to mosquitoes. Laboratory studies indicated that the susceptibility of *An. gambiae* s.s. to *P. falciparum* was decreased if mosquitoes had taken an uninfected blood meal before an infected blood (Vaughan *et al.*, 1994; Gass, 1977). This was hypothesized to be due to the accelerated digestion of blood meals in mosquitoes that had fed previously, which limits the time for ookinete transition through the midgut and development into oocysts (Vaughan *et al.*, 1994; Gass, 1977). However, another study found that both infection prevalence and oocyst number was higher in *An. gambiae* s.s. that had been fed two rather than one uninfected blood meal before an infectious blood meal containing *P. falciparum* (Okech *et al.*, 2004a), which the authors suggested could be due to the increased nutrition from blood promoting rather than decreasing parasite sporogonic success in vectors.

In natural infections, variations in host red cell density are known to also influence parasite transmission success through its association with gametocyte production and

density. Laboratory experiments have shown that adding lysed erythrocytes to parasite cultures can enhance gametocyte production (Schneweis *et al.*, 1991). Correspondingly, field studies have shown that PCV and malaria gametocyte densities are negatively correlated, with higher gametocyte densities being associated with low PCV blood (Drakeley *et al.*, 1999; Price *et al.*, 1999). These findings are consistent with laboratory rodent models (Ferguson *et al.*, 2003b; Paul *et al.*, 2004; MacKinnon & Read, 1999a) and other field studies (Drakeley *et al.*, 1999; Price *et al.*, 1999; Nacher *et al.*, 2002) that suggest parasite transmission success is higher from anaemic hosts.

The correlation between host anaemia and infectiousness to mosquitoes in natural situations may be due to the higher gametocyte density in these hosts. In addition, the reduced viscosity of low PCV blood may make it easier for mosquitoes to obtain large blood meals, and thus ingest a greater number of gametocytes (Daniel & Kingsolver, 1983; Shieh & Rossignol, 1992; Drakeley *et al.*, 1999; Gupta *et al.*, 1994; Taylor & Hurd, 2001; Dawes *et al.*, 2009). The effect of low PCV or anaemia on the per capita infectiousness of parasites to mosquito vectors (e.g. standardized for gametocyte density) is not yet known and will be experimentally investigated here.

1.3.3 Determinants of malaria parasite sporogonic success in mosquitoes

Once malaria parasites have successfully infected a mosquito, their further development (sporogony) onto the final human-infective stage (sporozoites) depends on the efficiency with which transitions between key parasite life cycle stages are made. During the first half of sporogony, ingested gametocytes form gametes, and then ookinetes which invade the mosquito midgut and develop into oocysts. This transition from gametocyte to oocysts results in high losses (Alavi *et al.*, 2003): only 0.2% of ingested *P. berghei* gametocytes become viable ookinetes, and of them, only 2-20% develop into mature oocysts (Alavi *et al.*, 2003). Similar losses have been documented through the macrogametocyte to oocyst stage in *P. falciparum* laboratory cultured gametocytes that were membrane-fed to *An. gambiae* (Vaughan *et al.*, 1992). Overall a net decrease of 2,754-fold was observed (an average of 40-fold decrease in transition from macrogametocyte to ookinete stage, and a 69-fold decrease in transition from ookinete

to oocyst stage) in *An. gambiae* mosquitoes infected with *P. falciparum* (Vaughan *et al.*, 1992).

Parasite numbers expand during the second half of sporogony when sporozoites mulitiply in oocysts, before being released into the haemolymph and infecting mosquito salivary glands. Ross originally reported that approximately 1000 sporozoites could develop in a *P. falciparum* oocyst (Ross & Thomson, 1910). Further studies with Asian vector *An. dirus* revealed mean sporozoite counts of 3,688 from *P. vivax* oocysts and 3,385 from *P. falciparum* oocysts respectively (Rosenberg & Rungsiwongse, 1991). However, a much higher count of 9555 sporozoites per oocyst was found in a wild-caught *An. funestus* in Northern Tanzania (Pringle, 1965), reflecting the high variability in sporozoite development between settings, parasite and vector species. In the laboratory, a linear relationship between oocyst density and salivary gland sporozoite densities has been documented (Vaughan *et al.*, 1992), with the majority of oocyst-infected mosquitoes developing sporozoite infections (Vaughan *et al.*, 1992). The situation in wild-caught mosquitoes may differ as sporozoites failed to enter the salivary glands in 43.5% and 10% of oocyst-infected *An. gambiae* sampled from 2 villages respectively (Lombardi *et al.*, 1987). However above a certain minimum threshold, sporozoite numbers are unlikely to limit mosquito transmission potential. This is because laboratory experiments have shown that the majority of *P. falciparum*-infected *An. gambiae* and *An. stephensi* mosquitoes transmit only 1-25 sporozoites during feeding (Beier *et al.*, 1991a), indicating that the number of sporozoites produced by even one small oocyst (<1000 sporozites) would be sufficient to initiate infections in more new hosts than a mosquito could bite in their lifetime.

A number of environmental and biological factors have been hypothesized to influence the successful development of malaria parasites through the sporogonic cycle. The first is competition for nutritional resources within mosquitoes. Malaria parasites must extract several nutritional resources from mosquitoes to complete their sporogony, including components of the mosquito haemolymph which are incorporated into growing oocysts (Warburg & Miller, 1992). There is some evidence that parasites obtain glucose from the haemolymph of mosquito vectors (Mack *et al.*, 1979; Atella *et al.*, 2006; Atella *et al.*, 2009). Furthermore, mosquitoes infected with rodent malaria oocysts consumed more glucose than non-infected mosquitoes (Mack *et al.*, 1979; Rivero & Ferguson, 2003), and

the oocyst density of *An. stephensi* infected with *P. yoelii yoelii* was positively correlated with concentration of glucose in the sugar water provided to them (Lambrechts *et al.*, 2006).

Some evidence of resource competition during sporogony comes from observation of density-dependent oocyst development. Studies have shown that oocyst size (an indicator of the number of sporozoites developing within them) is relatively uniform when the number of oocysts on a mosquito midgut is low (Beier *et al.*, 1990b; Beier *et al.*, 1991b; Lombardi *et al.*, 1987), but is very variable at high oocyst densities (Sinden & Strong, 1978a). This has been interpreted as evidence of competition for mosquito resources at high parasite densities, with the rapid growth of some oocysts suppressing the growth of others (Sinden & Strong, 1978a). Another limiting resource for parasite sporogony could be vertebrate blood. Evidence of this phenomenon is mixed. The number of sporozoites developing in oocysts of *An. dirus* was not increased by the provision of a second blood meal 7 days after infection, but mosquitoes who received this extra blood meal were 3.4 times more likely to develop salivary gland infections (Rosenberg & Rungsiwongse, 1991). A similar effect was reported after feeding uninfected blood to mosquitoes (*An. stephensi*) 5-11 days after an infective feed (Ponnudurai *et al.*, 1989). However, Ranford-Cartwright and Bell found that the number of sporozoites developing in *P. falciparum* oocysts could be increased only if four further uninfected blood meals were provided during sporogony (unpublished data, personal communication, Dr. L. Ranford-Cartwright). In contrast to these investigations of the influence of mosquito blood feeding frequency after infection on parasite sporogonic success, relatively little is known about how variation in the abundance and quality of blood resources within the infectious blood meal influences this process.

Finally, the abundance of key energetic resources within mosquitoes, such as lipids, sugars and proteins, could influence parasite development, by determining the availability of nutritional substrates for uptake by the developing parasites. Many reserves such as lipids and proteins are acquired primarily during larval development (Golberg & Demeillon, 1948; Timmermann & Briegel, 1999; Clements, 2000; Nishiura *et al.*, 2007), and are correlated positively with adult body size (Briegel, 1990b; Suwanchaichinda & Paskewitz, 1998; Takken *et al.*, 1998). However, laboratory investigations found no relationship between mosquito body size and the number of

oocysts or sporozoites that developed in *An. gambiae* s.s. (Humphreys, 2009). Environmental (e.g. temperature and pH) and biochemical (e.g. concentration of amino acids like valine, histidine and methionine in mosquito haemolymph) factors may also influence the outcome of sporogony in mosquito vectors (Beier, 1998), but much remains unknown about if and how these factors combine to influence malaria sporogony in mosquito vectors.

1.3.4 Determinants of malaria parasite virulence to mosquitoes

In ecological and evolutionary studies of parasite-host interactions, a reduction in host fitness as a result of infection relative to an uninfected host is defined as virulence (Stearns & Koella, 2008). Pathogen virulence may both promote transmission (if it is correlated with higher parasite replication rate) or decrease it (if the pathogen kills hosts before transmission is possible), giving rise to the 'trade-off' theory which predicts pathogen virulence evolves towards a level that most benefits their transmission (Frank, 1996). It has been generally predicted that pathogens should evolve towards low virulence in their insect vectors, because of the high dependence of parasite transmission on their survival (Day, 2002b). However, pathogen evolution towards zero virulence in insect vectors may not be possible if this would limit pathogen development and/or is unavoidable outcome of successful infection of mosquitoes (Read *et al.*, 1999; Frank, 1996).

Empirical studies have provided mixed results as to whether malaria parasites are virulent to their vectors or not. Virulence in terms of mosquito survival reduction due to infection has been the most thoroughly investigated, as this fitness trait has great impact on malaria parasite transmission. Some laboratory studies of rodent and avian malaria parasites have indicated that malaria infection reduces mosquito vector survival (Gad *et al.*, 1979; Klein *et al.*, 1982; Maier *et al.*, 1987; Dawes *et al.*, 2009; Ferguson & Read, 2002a; Ferguson & Read, 2002b; Ferguson *et al.*, 2003a). However, other studies, including those with human parasites and their natural vector species, have found no impact of parasite infection on vector survival (Boyd, 1940; Chege & Beier, 1990; de Buck, 1936; Gamage-Mendis *et al.*, 1993; Hogg & Hurd, 1997; Robert *et al.*, 1990). Studies of naturally-infected mosquitoes under field conditions have also yielded mixed results, with some suggesting potential for parasite virulence (Anderson & Roitberg, 1999; Lyimo &

Koella, 1992) and others not supporting this (Lines *et al.*, 1991; Hogg & Hurd, 1997). It has been hypothesised that the differing outcomes reported in these studies may be due to variations in study design, with virulence being substantially more likely to be observed in studies of unnatural vector-parasite associations that have not co-evolved together (Ferguson & Read, 2002b). Another explanation for these conflicting results could be that malaria parasite virulence to vectors is mediated by the nutritional quality of the infectious blood meal as will be investigated for the first time here.

Parasite virulence as reflected by a reduction in mosquito fecundity appears to be much more widespread and consistent than effects on mosquito survival. Most studies that have tested for an impact of malaria parasites on mosquito fecundity have reported a negative effect (Hacker & Kilama, 1974; Freier & Friedman, 1976; Hurd *et al.*, 1995; Hogg & Hurd, 1995a; Hogg *et al.*, 1995; Carwardine & Hurd, 1997; Jahan *et al.*, 1999; Ahmed *et al.*, 1999; Ferguson & Read, 2002a; Ferguson *et al.*, 2003b; Rivero & Ferguson, 2003; Briegel, 1990a). In addition to these effects on mosquito survival and reproduction, malaria parasites may influence other indirect determinants of mosquito fitness. For example, the distance flown by *An. stephensi* mosquitoes infected with sporozoites was significantly lower than for uninfected mosquitoes (Schiefer *et al.*, 1977). Thus there is good reason to predict that malaria parasites exert some degree of virulence on their mosquito vectors, even though the nature and magnitude with which this manifested is variable.

The degree of virulence which malaria parasites impose on mosquito vectors may depend on several external factors. Several studies in laboratory rodent models have shown that malaria parasite virulence varies between parasite genotypes (Ferguson *et al.*, 2003b), and may also be moderated by host genotype (Lambrechts *et al.*, 2005), environmental conditions such as nutrient availability [glucose] (Ferguson & Read, 2002a; Agnew & Koella, 1999; Lambrechts *et al.*, 2006) and mosquito age (Dawes *et al.*, 2009; Koella & Offenberg, 1999; Lines *et al.*, 1991). Another cause of variation in parasite virulence to mosquitoes could be vertebrate host factors such as red cell density which influences the resource value of infectious blood meals to mosquitoes. While such a phenomenon has been hypothesised, to date there have been no clear experimental investigations of the role of host haematological factors in promoting or mitigating parasite virulence to

mosquito vectors. A brief review of potential causes and mechanisms of parasite virulence to mosquitoes is needed to evaluate the scope for this.

1.3.4.1 Fitness costs of mosquito anti-parasite responses

It has been hypothesized that mounting an immune response against malaria parasites may incur a fitness cost in mosquitoes (Ferdig *et al.*, 1993). Furthermore, fitness costs associated with melanisation were demonstrated by comparing the reproduction of *Aedes* mosquitoes selected for refractoriness to infection and susceptible (non-melanising) lines (Yan *et al.*, 1997). However, similar results were not found in *An. gambiae* s.s. where those selected for refractoriness or susceptibility to *P. yoelii nigeriensis* had similar longevity and fecundity (Hurd *et al.*, 2005). Overall, the authors concluded that the nutritional costs of mounting an immune response may be less than those imposed on mosquitoes bearing an infection burden (Yan *et al.*, 1997; Hurd *et al.*, 2005).

1.3.4.2 Fitness costs due to parasite-associated pathology

Around 24-48 hours after ingestion of an infectious blood meal, malaria parasite ookinetes penetrate the mosquito midgut epithelial cells and this invasion can lead to the death and extrusion of invaded epithelial cells from midgut wall (Baton & Ranford-Cartwright, 2004; Baton & Ranford-Cartwright, 2007; Han *et al.*, 2000). The removal of damaged cells from the midgut epithelium presumably has a cost for mosquito body resources although this has not yet been formally demonstrated (Baton & Ranford-Cartwright, 2005a; Baton & Ranford-Cartwright, 2004; Baton & Ranford-Cartwright, 2007).

1.3.4.3 Fitness costs due to parasite resource depletion

Malaria parasite virulence to mosquitoes could also arise as a consequence of the depletion of key mosquito energetic resources by parasite growth. As reviewed above, there is evidence that oocysts developing in mosquito midguts take up a significant amount of lipids from mosquitoes (Atella *et al.*, 2006; Atella *et al.*, 2009). Furthermore, concentrations of amino acids such as valine, histidine, methionine, arginine, proline and asparagine have been reported to be significantly decreased in infected mosquitoes (Ball

& Chao, 1976). However, whether malaria parasites could reduce mosquito energetic reserves to the point where their fitness is compromised is unclear. If nutrient competition is the primary cause of malaria parasite virulence to mosquitoes, it would be predicted that mosquito fitness reduction increases with parasite burden. However, the mortality of very heavily infected mosquito has been frequently found to be no different to that of uninfected mosquitoes in many laboratory studies (Gad *et al.*, 1979; Maier *et al.*, 1987; Ferguson & Read, 2002b; Hurd, 2003; Schwartz & Koella, 2001; Hogg & Hurd, 1997; Chege & Beier, 1990; Gamage-Mendis *et al.*, 1993).

1.4 Specific hypotheses for the effect of host erythrocyte density and malaria parasite transmission

As outlined above, the central hypothesis for investigation in this study was that anaemia as characterized by a reduction in human red cell density (PCV) could have an impact on malaria transmission success by influencing mosquito vector fitness, their ability to become infected by malaria parasites, parasite sporogonic success in mosquitoes, and the virulence that mosquitoes experience from infection. As reviewed above, several ecological and mosquito nutritional factors are known to influence these mosquito and parasite traits. Here I present specific hypotheses for how human host anaemia may influence these determinants of malaria transmission. These predictions have been experimentally tested in the research presented in this thesis.

1.4.1 Erythrocyte density and mosquito vector fitness

Low red blood cell density as typical of anaemia could have an impact on mosquito fitness by affecting the size or protein content of the blood meal. The direction in which anaemia could affect mosquito blood meal size and/or protein intake, however, is not clear. On one hand, anaemia could reduce mosquito fitness as, for a given blood meal size, the protein content of anaemic blood is less than that of normal blood (Taylor & Hurd, 2001). On the other hand, it is possible that anaemia could enhance mosquito fitness by allowing bigger volumes of blood to be imbibed. For example as blood PCV falls, so does its viscosity, which may increase the rate at which blood can be imbibed, and possibly the total volume of intake (Shieh & Rossignol, 1992; Daniel & Kingsolver, 1983; Ribeiro *et al.*, 1985).

This potential 'feeding-rate' benefit of anaemic blood may be wiped out at very low red cell densities where the increased uptake can no longer compensate for the poorer erythrocyte density (Taylor & Hurd, 2001). Accordingly, mosquitoes fed on blood of very low PCV may have reduced energetic reserves. Thus, blood of intermediate PCV is proposed as the best resource for mosquito fitness (Daniel & Kingsolver, 1983). However in most previous investigations of this phenomenon, host anaemia has been created by malaria infections (Taylor & Hurd, 2001; Ferguson *et al.*, 2003b). Therefore, the impact of PCV separate from parasite infection has not been investigated yet. My prediction was that mosquitoes fed on blood of normal PCV (50%) would produce more eggs and survive longer than mosquitoes fed on blood with a PCV equivalent to severe anaemia (15% PCV).

1.4.2 Erythrocyte density and parasite infectiousness

There are two different ways that anaemia could influence parasite infectiousness to mosquitoes: 1) enhancing gametocytaemia in host blood, and/or 2) increasing the volume of host blood that mosquitoes are likely to consume from an infected host. The aim of this research is to test the second possiblity, that anaemic blood is in itself more likely to generate mosquito infections even with similar gametocyte densities. Anaemic individuals have a lower blood viscosity (Daniel & Kingsolver, 1983). As blood viscosity declines, mosquitoes can imbibe it at a faster rate which may result in a higher total volume consumed within a fixed period of time (Shieh & Rossignol, 1992; Taylor & Hurd, 2001). This could give rise to a higher probability of consuming enough gametocytes to start an infection in comparison to non-anaemic blood. Also, it is plausible that low PCV blood facilitates either fertilization and /or ookinete survival during the critical period of midgut invasion by the ookinete. For example, gametes in low PCV blood meal may be able to move around and find one another with greater efficiency due to its reduced viscosity. My prediction is that controlling for variation in gametocyte density, malaria parasites would be more likely to infect mosquitoes when transmitted in a blood meal of that equivalent to severe anaemia (15% PCV) than normal PCV (50%).

1.4.3 Erythrocyte density and malaria sporogonic success

So far, it is not known whether the PCV of the infectious blood meal has any longer-term impact on malaria sporogonic success within mosquitoes. In the absence of background information, it is predicted that parasite sporogonic development may be negatively

affected by low PCV in the infectious blood meal, as this could decrease the abundance of energetic reserves available for parasite development in mosquitoes.

1.4.4 Erythrocyte density and malaria virulence to mosquitoes

If malaria parasite virulence in mosquitoes is generated as a consequence of resource depletion, one prediction would be that parasite virulence to mosquito vectors is greatest when the red cell density of the infectious blood meal is lowest. In some previous studies, host anaemia appears to be associated with the degree of virulence observed in malaria-infected mosquitoes (Taylor & Hurd, 2001; Ferguson *et al.*, 2003b). Specifically, it was reported when host PCV decreased due to malaria infection, both mosquito blood meal size and fecundity were reduced (Taylor & Hurd, 2001; Ferguson *et al.*, 2003b). However, as host anaemia could not be disentangled from parasite density in these studies, it is difficult to know which factor was most responsible in generating virulence. My general prediction is that parasite virulence to mosquito vectors may be enhanced when the red cell density of the infectious blood meal is lowest.

1.5 Study system

This project was conducted using *An. gambiae* s.s mosquitoes that are reared under insectary conditions in the University of Glasgow. This species is a natural and important vector for transmitting malaria parasites such as *P. falciparum* in Africa (Bruce-Chwatt, 1985). Malaria parasite gametocyte culture and mosquito infection followed established protocols for human malaria parasite infection of mosquitoes (Carter *et al.*, 1993). One clone of *P. falciparum*, 3D7, was used in this work.

This thesis is separated into seven chapters:

Chapter 1: This chapter provides a general overview of malaria, the mosquito, parasite and host interactions on which it depends, and the potential role of host haematological factors in influencing transmission.

Chapter 2: This chapter describes the general methods used for the experimental studies conducted in this project.

Chapter 3: This chapter presents results from a pilot experiment conducted to test the suitability of laboratory conditions and protocols for measuring variation in the key mosquito fitness traits under study here (survival and fecundity). Specifically I tested whether variation in experimental conditions such as the human donor from which blood is obtained, and duration of blood storage prior to experimentation, had a significant impact of the mosquito fitness traits of interest.

Chapter 4: The chapter presents results of an experiment to test the hypothesis that human blood PCV influences the fitness of *An. gambiae* s.s. in terms of their survival and reproduction.

Chapter 5: This chapter presents results of experiment to test the hypothesis that host PCV influences the infectiousness of malaria parasites to *An. gambiae* s.s. vectors. My prediction was that malaria parasites would be more likely to infect mosquitoes when transmitted in a blood meal of that equivalent to severe anaemia (15% PCV) than normal PCV (50%).

Chapter 6: This chapter presents results of experiments to test the hypothesis that the PCV of an infectious blood meal influences the virulence that *An. gambiae* s.s. experience from malaria infection. My prediction was that parasite virulence to mosquito vectors would be greater when the red cell density of the infectious blood meal is equivalent to that of someone with severe anaemia (15% PCV) compared to blood of normal PCV (50%).

Chapter 7: In the final chapter, an overview of key research findings are presented, and their implications for malaria epidemiology, evolution and control discussed.

Chapter 2. General materials and methods

2.1 Mosquito rearing

Mosquitoes used in this project were from a laboratory colony of *An. gambiae* s.s (Keele line). *Anopheles gambiae* s.s. larvae were reared under standard insectary conditions of 27±1◦C, 70% humidity and a 12h light: 12h dark cycle. Larvae were fed *ad libitum* on TetraMin fish flakes (Tetra ltd., UK). Pupae were collected from the insectary stock and moved into a holding cage for emergence. The adults that emerged were fed *ad libitum* on a 5% glucose solution supplemented with 0.05% (w/v) 4-aminobenzoic acid (PABA).

2.2 Human blood and serum

Human blood and serum was obtained from the Glasgow and West of Scotland Blood Transfusion Service. Whole blood from donors of any blood group was provided in Citrate-Phosphate-Dextrose-Adenine (CPD_A) anti-coagulant/ preservative. Prior to use, the preservative and any remaining white blood cells were removed by washing the blood three times with incomplete RPMI medium (RPMI 1640 (Gibco) supplemented with 25mM HEPES buffer and 50mg/L hypoxanthine (Sigma)). The red blood cells were resuspended in incomplete medium to 50% v/v and then kept refrigerated at +4°C. Fresh blood was obtained on a weekly basis. Stocks of serum (off the clot) were obtained as frozen packs from different donors of blood group AB. Several packs were pooled and heat-inactivated at 56°C for one hour to remove complement before use. Serum was then stored at -80°C until use.

2.3 Preparation of the blood meal

Blood at different packed cell volumes (PCV) was prepared by diluting red cells in appropriate volumes of serum. Within an experiment, the same batch of serum was used for all different treatments. Washed erythrocytes were centrifuged at 1500 x *g* for 5 minutes to pellet the cells. The supernatant was then removed and the pellet resuspended in human serum to achieve two different PCVs representative of blood from a normal (normal PCV = 40-50%) and severely anaemic human host (low PCV <15%). The PCV of the prepared blood mixtures was checked prior to mosquito feeding by drawing a 30 µl sample into a 1.15 × 1.55 × 75 mm capillary tube (Hawksley and Sons Ltd, Lancing,

Sussex, England) and centrifuging for 5 minutes in a Hawksley microcentrifuge at 3300 x*g*. The resultant PCV was calculated as the percentage of the total volume of the capillary tube that consisted of packed red cells rather than serum. Additionally, a small sample of the blood (10 µl) from each experimental treatment was analysed using a HemoCue reader (Hemocue Ltd., Derbyshire, UK) to measure the haemoglobin concentration before feeding to mosquitoes in all experimental replicates.

2.4 Membrane feeding

Three to five days post emergence, adult females were collected and transferred into cardboard holding pots (rim 90mm X height 110mm) sealed with netting on top in preparation for blood feeding (Figure 2.1). Mosquitoes were held in these pots for a further two days before blood feeding and provided with glucose / PABA solution *ad libitum*. The day before the blood feed, the glucose was removed and the mosquitoes were provided with distilled water only to increase their willingness to take a blood meal through the membrane feeder.

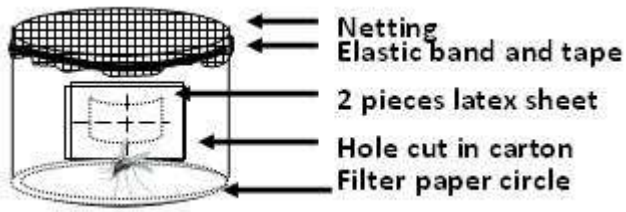

Figure 2.1. Structure of mosquito pot

Membrane feeding was carried out following established protocols (Carter *et al.*, 1993). Glass membrane feeders covered with Goldbeaters skin (ZH de Groot, Heemraadssingel 255a, 3023CE, Rotterdom, The Netherlands) were attached to base of glass membrane feeders using elastic bands, and the feeders were connected to a circulating water bath at 37◦C. A 1 - 1.5ml volume of the blood mixture (Section 2.3) was placed into each membrane feeder, which was then lowered onto the surface of a holding pot containing pre-starved *An. gambiae* s.s. Mosquitoes were allowed to feed from the membrane feeder for 15-20 minutes. Two to three hours after the membrane feed, pots were inspected and all unfed mosquitoes were removed and killed by freezing. Individual fed mosquitoes were transferred into individual 7ml plastic bijou tubes (one per tube) which

were labelled with a unique identifier code to designate their experimental treatment. These mosquitoes were maintained under standard insectary conditions and given access to a 5% glucose solution containing 0.05% PABA solution through a cotton wool pad placed on the netting that sealed the top of the tube. Pads were changed every day.

2.5 Measuring mosquito blood meal size

The amount of blood (erythrocytes) taken by individual mosquitoes during feeding was estimated by measuring the amount of haematin excreted after blood feeding, which is correlated with the total mass of erythrocytes imbibed (Briegel, 1980). To measure haematin excretion, individual mosquitoes were kept in separate tubes for three days after feeding to allow blood digestion to be completed. After 3 days, each mosquito was transferred into a new tube (7ml) bearing the same ID number and details, and the haematin deposited in their previous tube was used to estimate blood meal size following Briegel, 1980. In brief, 1ml of a 1% (w/v) Lithium carbonate solution was added to each tube and mixed well to dissolve the haematin. The absorbance of the resultant solution was measured at 405 nm in an ELISA plate reader (Dynex Ltd., MRX Revelation, San Diego, CA, US) which had been calibrated against a lithium carbonate-only blank. The amount of haematin in the sample was estimated by comparison to a standard curve prepared using porcine haematin (Sigma) at concentrations of 1 to 30µg/ml. The blood meal size of each sample (mass of haematin) was calculated from the regression equation obtained from the standard curve.

2.6 Measuring mosquito fecundity and oviposition rate

After haematin collection, mosquitoes were transferred into new plastic tubes (7ml) that contained water to a depth of 1 cm to allow for oviposition. The next day, all tubes were inspected for eggs. If eggs were present, the mosquito was moved into a new tube, and the number of eggs laid counted under a dissecting microscope (fecundity). Oviposition rate was calculated as the proportion of the blood fed mosquitoes that laid eggs when moved into oviposition tubes three days after the blood feed.

2.7 Survival and body size of adult mosquitoes

Each day after blood feeding, tubes were examined to check whether mosquitoes were alive, and record their day of their death. After death, the body size of mosquitoes was estimated by measuring their wing-length (a standard indicator of body size) (Briegel, 1990b). One wing was dissected from the body and placed in a drop of distilled water on a microscope slide. The length of the wing was measured using a digital camera imaging system (Moticam 2300) that was connected to the microscope eyepiece. Wing length was measured as the distance from the axillary incision to the apical margin (Nasci, 1986) using precalibrated software (Motic Images Plus, v. 2.0), (Figure 2.2).

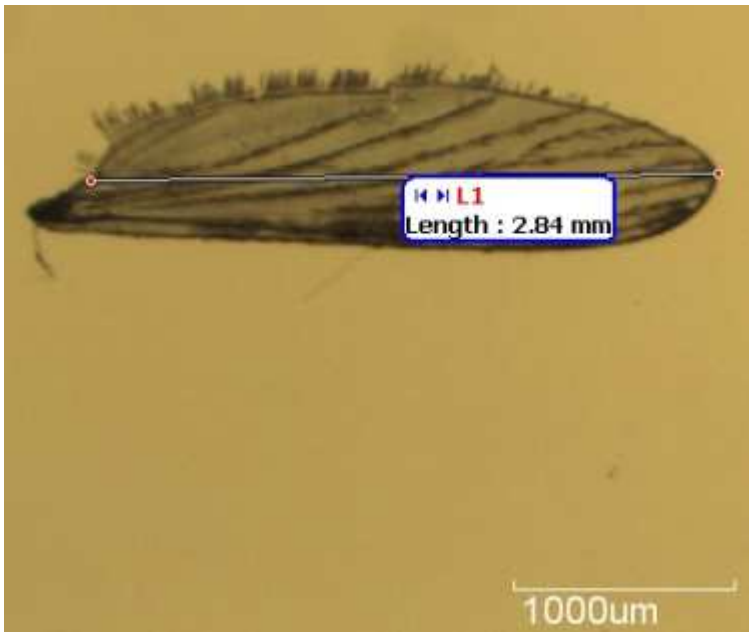

Figure 2.2. Mosquito wing length, which is measured as the distance from the axillary incision to the apical margin using precalibrated software and a digital camera.

2.8 Statistical analysis

Statistical analyses were conducted to evaluate the effect of response variables related to the specific experimental hypotheses of this project on key indicators of mosquito and parasite fitness. Throughout this project, mosquitoes were exposed to different experimental treatments in groups, as defined by the number that were provided blood from the same membrane feeder (up to 130). Replicates were conducted by feeding numerous groups of mosquitoes with blood from the same experimental treatment

(provided in different membrane feeders). Consequently individual mosquito fitness traits could be influenced both by fixed experimental treatments, and the random effect of feeding group replicate. Thus it was decided that all data should be analysed using the mixed models approach which allows incorporation of both fixed and random effects (Laird & Ware, 1982; Mclean *et al.*, 1991; Burnham & Anderson, 1998; Burnham *et al.*, 2011). All analyses were conducted using the R statistical software (v.2.10.1 or v.2.12.2 (Crawley, 2007).

Three major classes of response variables were investigated in these studies: (1) binomial data (probability of oviposition and infection prevalence), (2) continuous data following a normal distribution (blood meal size, number of eggs laid), and (3) continuous data that were not normally distributed (e.g. number of days that mosquitoes survived, and numbers of oocysts and sporozoites per infected mosquito). A similar approach was adopted to investigate the significance of fixed (PCV, malaria infection) and random (replicate) experimental treatments for all these outcome variables, with different statistical models used as appropriate for the nature of their underlying distribution. The generalised linear mixed effect model procedure (GLMM, package= lme4) in the R statistical software (v.2.12.2 (Crawley, 2007)) was used to analyse binomially-distributed response variables. The general linear mixed model procedure (package= lme4) was used for analysis of continuous variables. Survival data was analysed using the proportional hazards model as described below. The other continuous response variables of oocyst and sporozoite numbers were best described by a negative binomial distribution, and analyzed using this model as described below.

2.8.1 Model selection and statistical significance

The statistical significance of the fixed explanatory variables were tested using the model selection procedure (Burnham & Anderson, 1998). In Chapter three, the main explanatory variables of interest were blood donor , and the duration of blood storage prior to use. In chapters four and five, blood PCV was the primary explanatory variable. Finally in Chapter six, the key explanatory variables tested were blood PCV, blood infection status (*P. falciparum*-infected or control) and their interaction.

In brief, both forward and backward model selection procedures were conducted for each analyses, with the final model 'best' statistical model being the one concluded to be statistically significant through both approaches. As in all cases, forward and backward selection identified the same statistically significant model, for simplification all results are described in the context of backward elimination which is generally assumed to be the most robust (Crawley M.J., 2007).

For forward selection, the significance of each potential explanatory variable and their interactions were evaluated by sequentially fitting them to a null model that contained only the random effect of replicate, and performing the likelihood test (LRTs) to assess whether their addition led to a statistically significant improvement by decreasing the negative log likelihood of the model ($P< 0.05$). In cases where more than one explanatory variable led to a statistically significant improvement over the null, the Akaike Information Criteria (AIC) was used to compare the strength of statistical support for alternative models on the basis of their weight (*w*AIC) which can be used as a measure of the amount of information explained by particular statistical model relative to the total information (Burnham & Anderson, 1998). The "best model" was identified by comparison of their AIC and estimated Akaike weights w_i (Burnham & Anderson, 1998) The model with the greatest strength of statistical support (as evaluated by *wAIC*) was selected as the "best model". Once the 'most significant' variable had been selection, a second round of selection was carried out to test whether the further addition of remaining explanatory variables and/or their interaction could significantly improve the explanatory power of this model. Following this procedure, a series of nested models were generated (with the null model as the base), in which one more term was added at each step (first all main effects, then their interactions). Likelihood Ratio Tests (LRTs) were used to evaluate the significance of each added term (using the ANOVA procedure in R).

The backward elimination model selection procedure started with the most complicated model (maximal model) which contained all fixed effects and their interactions of interest, and random effects. This model were simplified by step wise elimination of non significant effects until a minimal statistical model which contained only significant effects was retained (Crawley M.J., 2007).

2.8.2 Survival analysis

Survival analysis was used to examine the differences in the time till mosquito death. The Proportional hazard model was the basic model used to analyse survival data (also known as the Cox regression model (Cox, 1972)). The Cox regression model is 'semi-parametric' in that it contains both parametric and nonparametric effects. In the context of this survival analysis, the 'semi-parametric' effect arises from the fact that no specific distribution is assumed for survival data.

The Cox proportional hazards model procedure (coxph) in the R statistical software (v.2.10.1 or v.2.12.2 (Crawley, 2007)) was used to estimate the impact of different explanatory variable on the survival of *An. gambiae* s.s.. In this model, the random effect of 'replicate' was incorporated by fitting a frailty function (Hougaard, 1995). In these models, fixed effects and their interaction were fit to a base model including the random effect of experimental replicate. The backward elimination procedure was used to sequentially remove non-significant terms to reach the minimal statistically significant model.

2.8.3 Negative binomial model

Parasite abundance data (oocyst and sporozoites numbers) typically follow a negative binomial distribution, with most individuals having low or no parasites, and a few having very high loads (Pringle, 1965; Medley *et al.*, 1993; Ichimori, 1989; Rosenberg & Rungsiwongse, 1991; Gamage-Mendis *et al.*, 1993; Vaughan *et al.*, 1994; Vaughan, 2007). The two parasite abundance measures estimated here, oocyst number (oocyst intensity) and sporozoite load (the total number of malaria parasite genomes per mid-gut) were both distributed in this manner (Crawley, 2007; Bell *et al.*, 2005; Bell & Ranford-Cartwright, 2004). Thus to test for statistical differences in these variables between experimental treatment, the negative binomial model was used ('glm.nb' procedure in the R statistical software, v.2.10.1/2.12.2, (Crawley, 2007)). The negative binomial model has the same general structure as a Poisson distribution but with the extra parameter "k" to model over-dispersion.

At present it is not possible to fit a negative binomial distribution within the generalized linear mixed model procedures in the R statistical software package. Here and in most

other software packages, the negative binomal model can be applied onto to general linear models that include just fixed effects. Thus for these analyses, 'experimental replicate' was incorporated as a fixed treatment effect. This allowed the significance of the main variables of interests being tested while controlling for variation due to experimental replicate. Although this approach requires more degrees of freedom to be allocated to the experimental replicate effect, the results it will generate for the main treatment effects are expected to be very similar to obtained from a random effects model. The significance of fixed effects was estimated by stepwise elimination procedures as described above.

2.8.4 Controlling for additional sources of variation and reporting

In all analyses, the effect of the main experimental effects and their interactions were investigated while controlling for variation in mosquito body size (as indexed by wing length) as the latter variable is known to generate considerable variation in mosquito feeding and fitness (Briegel, 1990a; Timmermann & Briegel, 1999). Thus failure to account for naturally occurring variation in body size between mosquitoes might confound ability to test the significance of the main experimental effects.

For reporting of all results, the significance of all explanatory effects were evaluated by using likelihood ratio test (LRT). All chi-square values "X^2" which were reported attribute to the output of linear mixed and generalised mixed models. Values called as "OR" were odds ratios from Logistic regression and Cox proportional hazard models.

Chapter 3. The impact of blood storage time and donor on mosquito fitness

3.1 Aims and objectives

This chapter describes the results of a pilot investigation which aimed to investigate how variation in the human blood provided to mosquitoes in the laboratory influenced their fitness. The two main factors investigated were the human donor from which blood was obtained, and the duration of blood storage prior to experimentation. The impact of these factors on the fitness and feeding success (measured as blood meal size, fecundity and survival) of the major vector of African malaria *An. gambiae* s.s were examined under standard laboratory conditions, following blood-feeding using artificial membrane feeders. The results of this experiment provide a baseline for my subsequent work on mosquito fitness. I then moved onto experiments that address the core hypotheses of my thesis.

The key questions addressed in this chapter were:

1. Does the fitness of *An. gambiae* s.s. (as measured by blood meal size, fecundity and adult survival) vary when erythrocytes are provided from different human hosts?

The hypothesis being tested is that there is no significant difference in mosquito fitness between blood meals provided from different human donors. Under the null hypothesis, there should be no difference in longevity of adult mosquitoes, and no difference in the numbers of eggs laid, in mosquitoes which fed on human blood from three different donors.

2. Does the length of time that human blood has been stored prior to experimentation influence the fitness of the *An. gambiae* s.s. mosquitoes that feed on it?

The second hypothesis being tested is that blood storage time does not have an influence on mosquito fitness as measured by adult survival and egg production. Under the null hypothesis, there was no expected difference in survival and egg production in mosquitoes which fed on human blood stored for three different periods of time (7, 14, 21 days prior to experimentation).

3.2 Introduction

In the laboratory, interactions between parasites of humans and their arthropod vectors are generally studied through use of an *in vitro* experimental system in which vectors are infected using a membrane feeding device. Typically blood is obtained from healthy human volunteers who donate to a blood transfusion service, and is stored for various periods of time (freshly donated blood is stored up to six weeks before it is considered outdated for transfusion purposes (National Blood Service, 2005; Antonelou *et al.*, 2010)). The identity of donors providing samples to the blood bank is unknown and changes regularly, and the length of time blood is kept in storage prior to experimentation also varies due to demand. It is generally assumed that variation between blood donors and blood storage age has negligible impact on the fitness and infection dynamics of common vector groups such as mosquitoes, and thus it is assumed that these factors do not have to be standardized for routine experimentation. However, before embarking on the primary thesis research investigating the role of haematological variation on mosquito and parasite fitness, these critical assumptions that the source and age of the blood has no major impact on mosquito fitness were tested.

3.3 Methods

Blood from each of 3 different human donors was fed to *An. gambiae s*.s. mosquitoes at 3 time points: after 7, 14, and 21 days of storage at 4^{o}C. Within each donor and storage-time combination, blood was fed to three separate groups of approximately 210 female *An. gambiae* mosquitoes. Blood feeding followed standard methodology as described in Chapter 2 (sections 2.2, 2.4). Three batches of blood were obtained in the same week from the Glasgow and West of Scotland blood transfusion service and blood meals prepared at a packed cell volume representative of blood from a normal, healthy host (40-50%), which was confirmed by capillary tube centrifugation and hemocue, all according to standard methodology (section 2.3). Mosquitoes were offered a bloodmeal through membrane feeders as described (section 2.4) and unfed mosquitoes removed. Blood-fed mosquitoes were transferred from pots into individual 30ml plastic Universal tubes which were labelled with a unique identifier code, and the experimental treatment (donor, age of blood). These mosquitoes were maintained in the insectary and offered glucose as described (section 2.4). The amount of blood (erythrocytes) taken by

individual mosquitoes during feeding was estimated by the amount of haematin excreted (Briegel, 1980) as described (section 2.5). Mosquitoes were transferred to separate new plastic tubes for oviposition (section 2.6). Survival of mosquitoes was monitored until the natural day of death (section 2.7), and body size was estimated after death (section 2.7).

3.3.1 Calculation of sample size

Sample size calculations were conducted to estimate the number of mosquitoes needed in each experimental treatment in order to be able to detect a statistically significant difference in the fitness traits measured. Specifically, sample size calculations were performed on the basis of the mosquito fitness trait that was assumed to be the most influenced by haematological variation: fecundity (number of eggs laid per individual). Full details of the sample size calculations are provided in appendix 8.1).

Briefly, the number of mosquitoes required to detect a statistically significant difference in fecundity (number of eggs that laid per individual) was estimated on the basis of the following formula:

$N = [t]^2 \times 2 \times \text{variance} / (\text{difference to be detected})^2$ (Van Emden, 2008)

Data obtained from colleagues working with *An. gambiae* s.s indicated the mean number of eggs laid per mosquito = 60 (SE = 4.1, Variance = 31.8, I. Lyimo pers. Comm.). The minimum difference in mosquito fecundity to be detected between treatments decided to be 10%, equivalent to a difference of 6 eggs assuming the above mean. The critical t-values [t] that need to be exceeded to reject a null hypothesis of fecundity were obtained from critical t- values table based on 4 degrees of freedom (arising from 2 experimental factors (blood donor and blood storage age), each with three levels) was calculated as $t_{p\ 0.05,\ 4} = 2.776$ - values above this indicate that the null hypothesis can be reliably rejected with a 95% probability) and $t_{p\ 0.10,\ 4} = 2.132$ - data producing t-values above this indicate we could correctly accept Ho with 90% probability of being right (90% power of the test). In this calculation, sample size is estimated as guided by two critical t values, the t- value for probability of α error (*P*- value = 0.05) plus that for ß error [at the required level of power 90%] (*P*- value = 0.1).

The calculation of sample size was then calculated from the formula:

$$N= [2.776 + 2.132]^2 \times 2 \times 31.8 / (6)^2 = 1532/ 36= 42$$

Previous studies in the group indicated that approximately 80% of female *An. gambiae* s.s. feed when offered a blood meal from a membrane feeder, and of those, approximately 80% go on to lay eggs (I. Lyimo, personal communication). Thus to account for the proportion of females that do not feed or lay eggs, 70 females were offered a blood meal in each treatment group (donor, blood storage age), from which 45 should lay eggs after blood feeding to meet the minimum sample size per treatment. Each treatment was repeated in triplicate.

3.3.2 Statistical analysis

The effect of the explanatory variables of blood donor source and storage time on the mosquito fitness traits of blood meal size, fecundity, oviposition rate and survival were tested using Generalised Linear Models as described in section 2.8. Mosquito body size (wing length), blood donor and blood storage time were fitted as fixed explanatory variables. Experimental replicate (three per treatment combination) was fitted as a random effect. The significance of each of the three potential explanatory variables was evaluated as described (section 2.8.1), by sequential removal of non-significant terms from the maximum model including all treatment effects, their interactions, mosquito body size, and the random effect of replicate.

3.4 Results

3.4.1 Effect of blood donor and storage time on mosquito blood meal size

The proportion of mosquitoes which took a blood meal varied from 94-100% (Appendix 8.2). Mosquito body size varied in these experiments (Figure 3.1- Figure 3.3, for blood of 7, 14 and 21 days storage time respectively), and as expected was positively related to blood meal size (Figure 3.1- Figure 3.3).

Initial analysis indicated that mosquito blood meal size was dependent on the 3-way interaction between blood donor, storage time and mosquito wing length (X^2_6 =14.01, *P*=

0.03). Consequently for ease of interpretation, separate analyses were conducted on data from each donor to test the effect of blood storage time, and separate analyses were conducted on data from each blood storage time to test the effect of blood donor.

There was significant variation in mosquito blood meal size between donors in trials with blood stored for 7 days (X^2_2 = 30.37, P< 0.001,Figure 3.1). Specifically, with 7-day old blood, mosquitoes acquired smaller blood meals (or less haemoglobin) from donor A than B or C (Figure 3.1). However, for 14 days old blood, mosquitoes acquired blood meals from donor A and B were roughly similar, and mosquitoes fed on donor C blood had the smallest blood meal size (day 14: X^2_2= 34.62, P < 0.001,Figure 3.2). With blood stored for longer periods, there were significant interactions between blood donor and mosquito body size (day 21: X^2_2= 19.75, P < 0.001 ,Figure 3.3), such that no one donor was consistently better or worse than others with respect to mosquito blood meal size.

Blood storage age was significantly related to mosquito blood meal size in trials with two out of the three donors (Donor A: X^2_1= 1.08, P= 0.58,Figure 3.4; Donor B: X^2_1 = 36.36, P< 0.001,Figure 3.5; Donor C: X^2_1= 7.74, P= 0.02,Figure 3.6). Generally, blood stored for the least amount of time (7 days) was associated with the largest blood meal sizes in trials with donors B and C (Figure 3.5- Figure 3.6).

Within each donor group, there was also a consistent positive relationship between blood meal size and mosquito body size (Donor A: X^2_1= 80.87, P < 0.001, Donor B: X^2_1= 113.46, P < 0.001; Donor C: X^2_1= 68.34, P < 0.001). Within one donor group (C), there was also a significant interaction between blood storage age and mosquito body size (X^2_2= 7.74, P= 0.02). In trials conducted with this donor, the slope of the relationship between mosquito blood meal size and body size was greater in blood stored for 7 days than for 14, or 21 days.

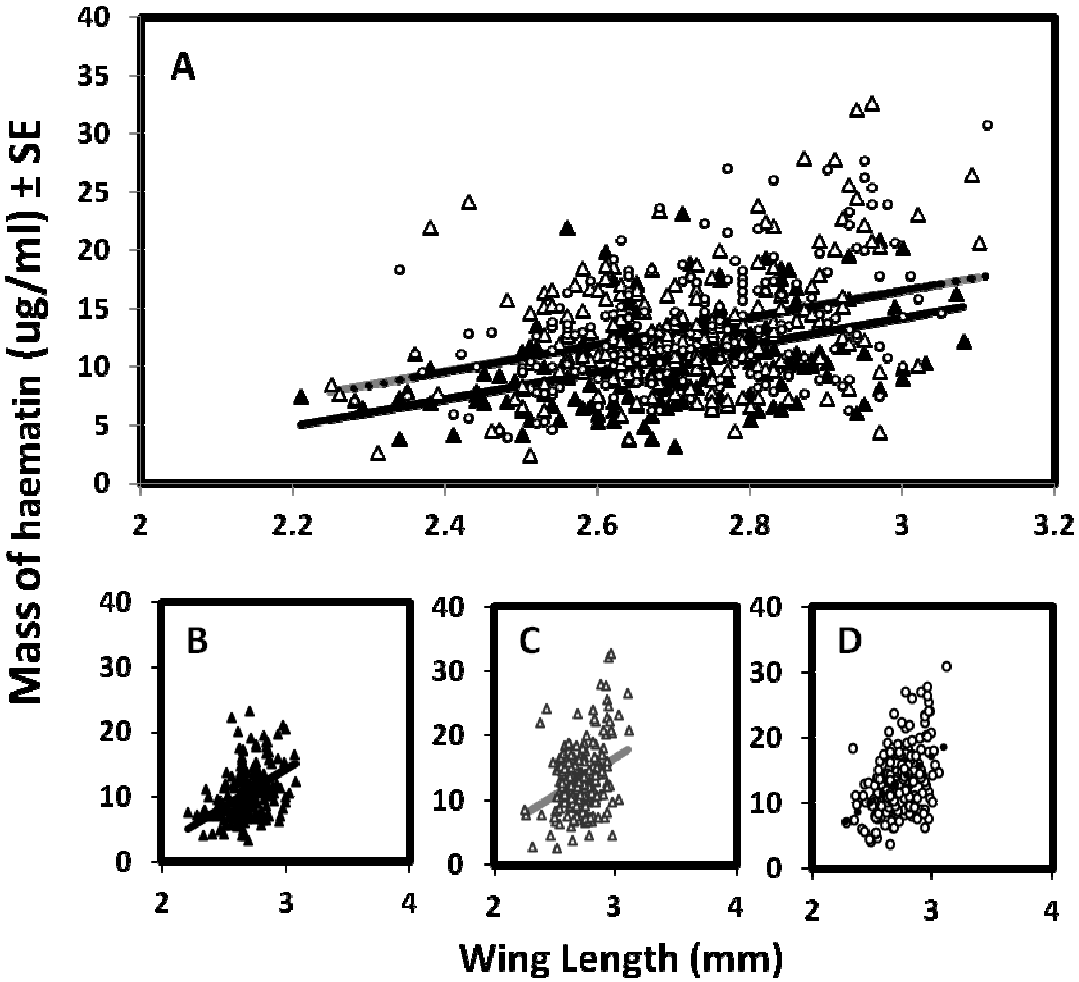

Figure 3.1. Relationship between mosquito body size (as indicated by wing length) and size of blood meal (measured as mass of haematin) for three different blood donors with blood stored for 7 days: Blood from donor A is shown as black triangles (▲), from donor B as white triangles (Δ) and from donor C as white circles (o). Panel A shows results from all three donors, and panels B-D show the data from each donor separately. The solid lines represent the regression line of best fit. In panel A, the line for donor A blood is solid black, for donor B blood and donor C blood, the regression lines overlap and so are not clearly distinguishable from one another.

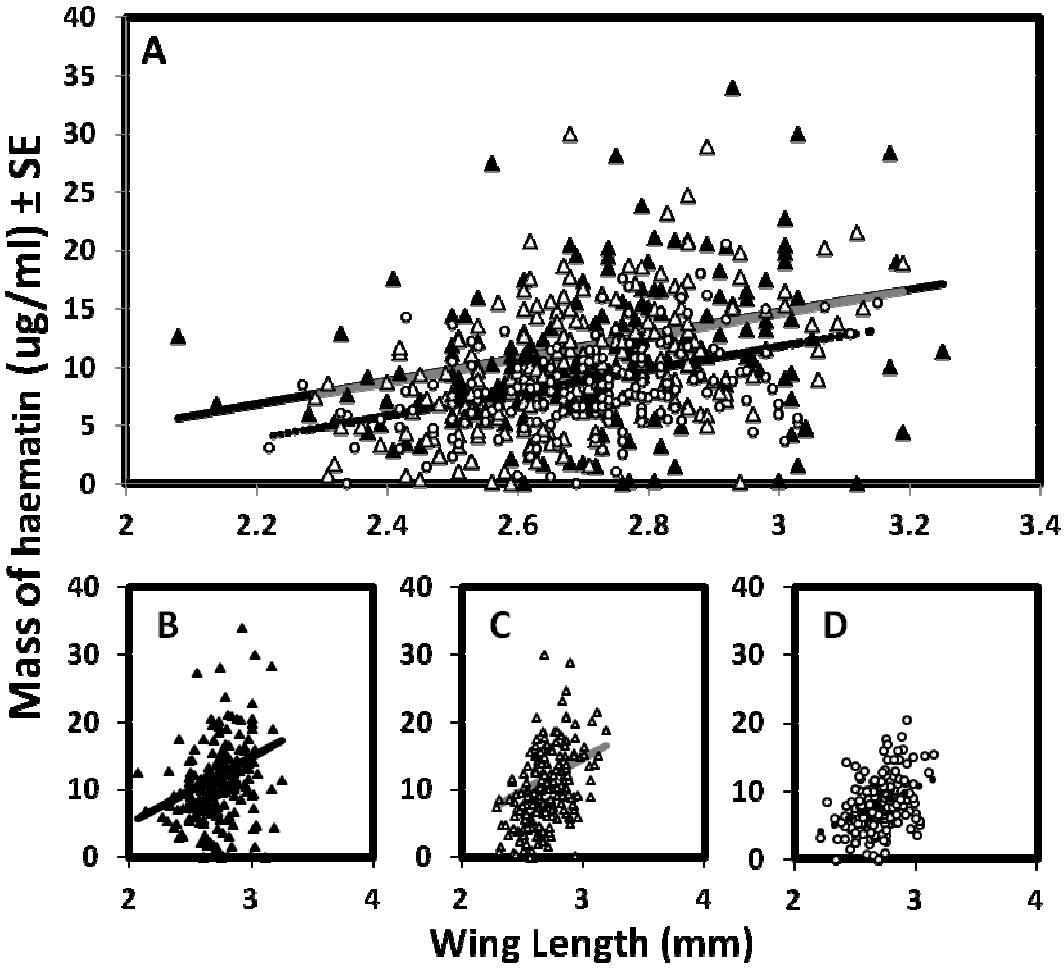

Figure 3.2. Relationship between mosquito body size (as indicated by wing length) and the size of their blood meal (measured as mass of haematin) for three different blood donors with blood stored for 14 days. Blood from donor A is show as black triangles (▲), from donor B as white triangles (Δ), and from donor C as white circles (o). Panel A shows results from all three donors and panels B- D show the data for each donor separately. The solid lines represent the regression line of best fit. In panel A the line for donor A blood is solid black, for donor B blood solid grey, and for donor C blood dashed black.

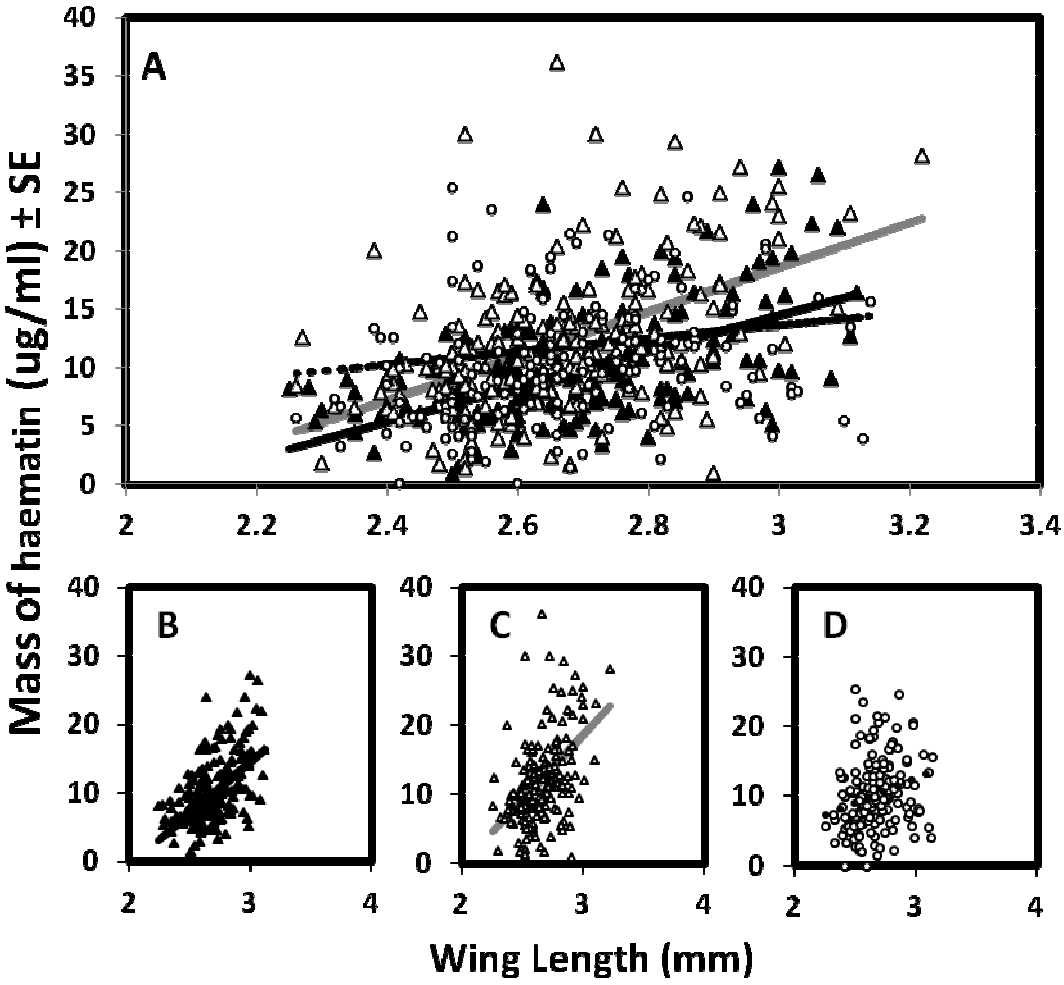

Figure 3.3. Relationship between mosquito body size (as indicated by wing length) and the size of their blood meal (measured as mass of haematin) for three different blood donors with blood stored for 21 days: Donor A blood is shown as black triangles (▲) donor B blood as white triangles (Δ), and donor C blood as white circles (o). Panel A shows results from all three donors and panels B- D show the data for each donor separately. The solid lines represent the regression line of best fit. In panel A the line for donor A blood is solid black, for donor B blood solid grey, and for donor C blood dashed black.

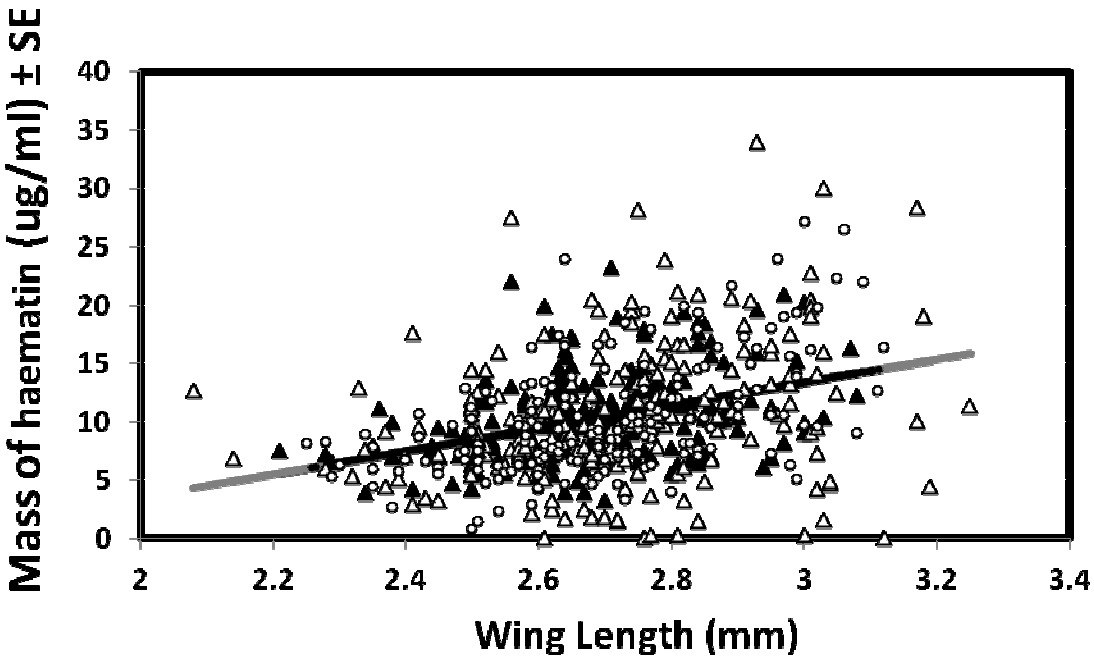

Figure 3.4. Relationship between mosquito body size (as indicated by wing length) and the size of their blood meal (measured as mass of haematin) for three different blood storage times with blood from donor A: 7 day old blood is shown as black triangles (▲) 14 day old blood as white triangles (Δ) and 21 day old blood as white circles (o). The solid lines represent the regression lines of best fit. Three regression lines are overlapped.

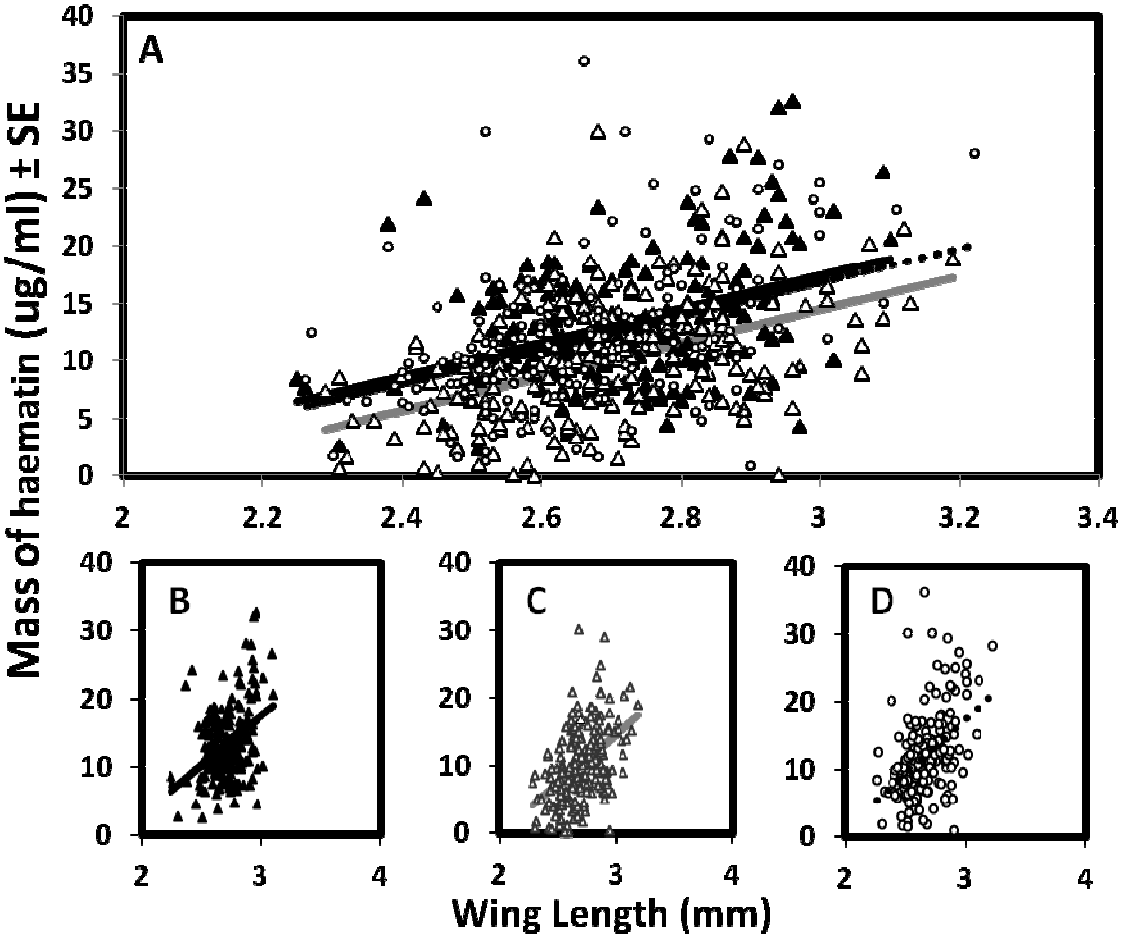

Figure 3.5. Relationship between mosquito body size and size of blood meal (measured as mass of haematin) for three different blood storage times with blood from donor B: 7 day old blood is shown as black triangles (▲), 14 day old blood as white triangles (Δ), and 21 day old blood as white circles (o). Panel A shows results from all three blood storage times, and panels B-D show the data for each donor separately. The solid lines represent the best fitted regression line. In panel A the line for 7 day old blood is solid black, for 14 day old blood solid grey, and for 21 day old blood dashed black. 7 day old blood and 21 day old blood lines cannot be distinguished easily as they almost overlap.

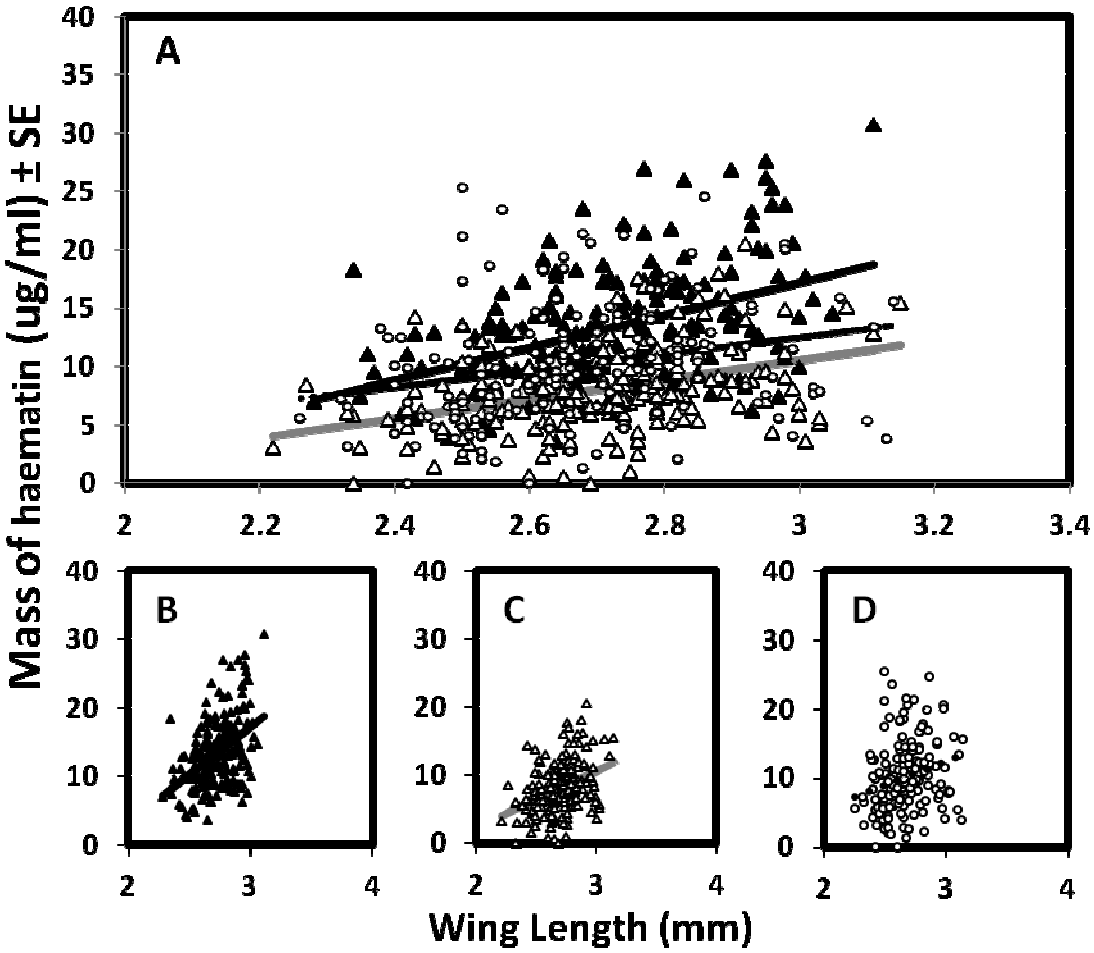

Figure 3.6. Relationship between mosquito body size and size of blood meal (measured as mass of haematin) for three different blood storage times with blood from donor C: 7 day old blood is shown as blank triangles (▲), 14 day old blood as white triangles (Δ), and 21 day old blood as white circles (o). Panel A shows results from all three blood storage times and panels B-D show the data for each donor separately. The solid lines represent the best fitted regression line. In panel A the line for 7 day old blood is solid black, for 14 day old blood solid grey, and for 21 day old blood dashed black.

3.4.2 Effect of blood donor and storage time on mosquito oviposition rate

The proportion of mosquitoes that laid eggs after blood feeding (oviposition rate) ranged from 29.9% to 68.6 (Appendix 8.2). Mosquito oviposition rate was not dependent on blood donor (X^2_2 = 5.30, *P*=0.07). However, both blood storage time and mosquito wing length (X^2_1 = 9.02, *P*= 0.002) had significant, independent impacts on oviposition rate. Specifically, mosquitoes had a higher oviposition rate after feeding on blood stored for 7 days than on that stored for 14 and 21 days (X^2_1 = 9.02, *P* < 0.001,Figure 3.7). Mosquito body size was positively related to oviposition rate, with the odds of mosquitoes laying eggs increasing by 86% for every 1mm increase in wing length (Odds Ratio= 0.86, 95% CI= 2.68-2.70).

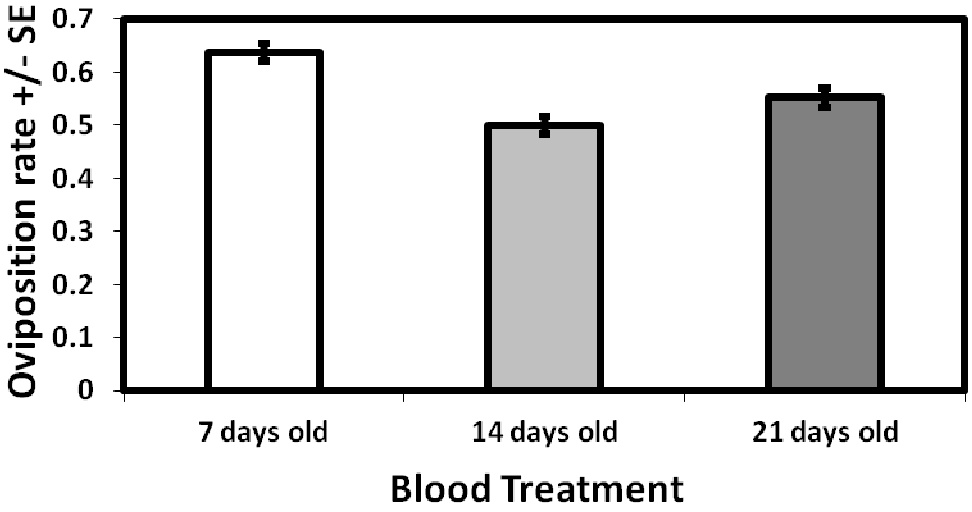

Figure 3.7. Oviposition rate of mosquitoes feeding on blood stored for different lengths of time. Bars show the estimated β value for each blood storage time which is estimated by logistic regression and error bars are S.E. of blood treatment which are obtained from the blood storage time model.

3.4.3 Effect of blood donor and storage time on mosquito fecundity

Mosquitoes which did not lay any eggs at all were excluded from the analysis of fecundity. There was no significant variation in mosquito fecundity between blood from different donors (X^2_2= 2.48, *P*= 0.29). However, mosquito fecundity was influenced by the interaction between blood storage time and mosquito wing length (X^2_2= 7.32, *P*= 0.02,Figure 3.8). While mosquito fecundity was positively correlated with wing length, the interaction of this variable with blood storage age indicates that there was no one storage age that was consistently best for mosquito fecundity.

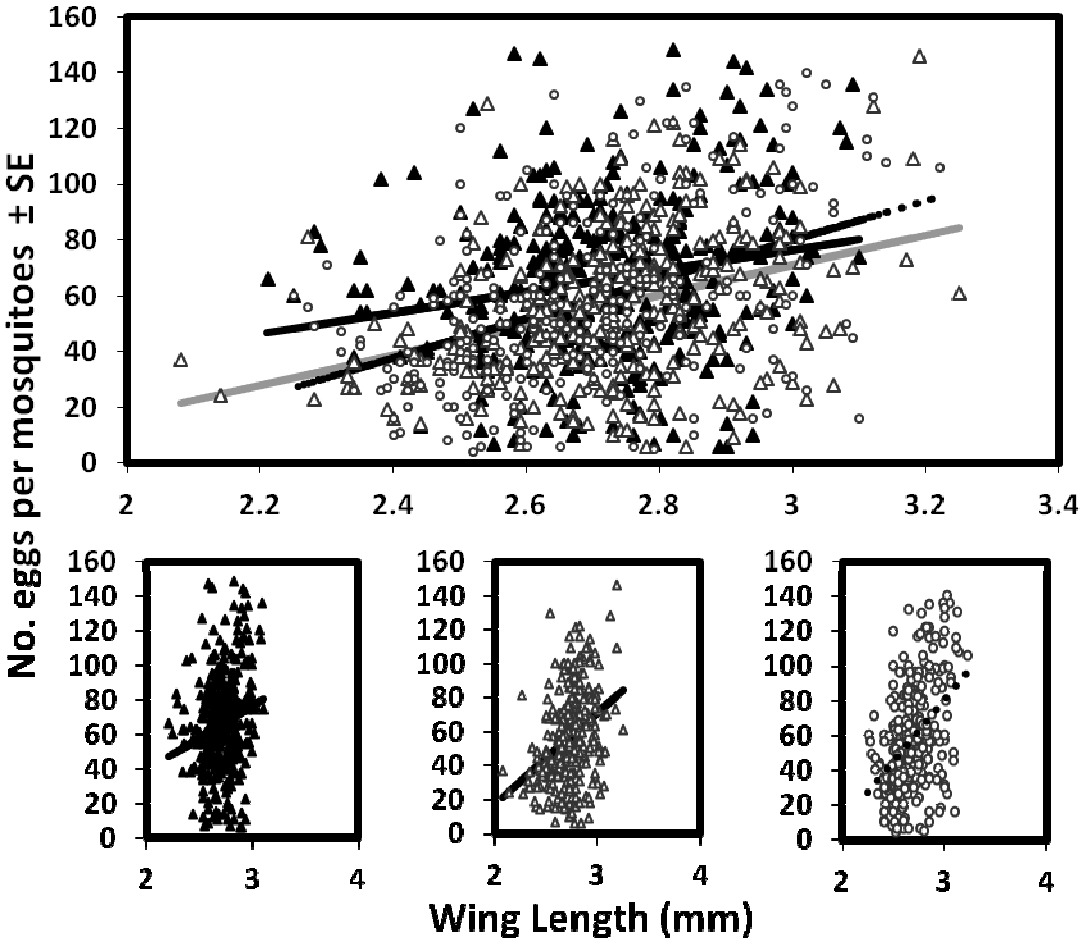

Figure 3.8. Relationship between mosquito body size and fecundity, for mosquitoes fed blood that had been stored for 7 (black triangles), 14 (white triangles) and 21 days (open circles) respectively. Panel A shows results from all three blood storage times, and panels B-D show the data for each blood storage time separately. The solid lines represent predicted regression lines from the statistical analysis. In panel A, the line for 7 day old blood is solid black, for 14 day old blood, is solid grey and for 21 day old blood, is dashed black.

3.4.4 Effect of blood donor and storage time on mosquito survival

Neither the length of time that blood was stored prior to feeding (X^2_2= 0.52, *P*= 0.76) nor blood donor (X^2_2= 2.95, *P*= 0.23) were found to have a statistically significant impact on mosquito survival. However, mosquito survival was significantly correlated to their body size (X^2_1= 17.17, *P*<0.001). Specifically, there was a predicted fall in the odds of mortality of 54% for every 1 unit increase in mosquito wing length [1mm] (OR=0.54 and 95% CI = 0.405-0.724).

3.5 Discussion

The experiments presented in this chapter examined the effect of blood donor and length of storage time of blood on the fitness of *An. gambiae* s.s feeding on it under standard laboratory conditions. In all experiments, the mass of blood that mosquitoes obtained from feeding, as measured by the mass of the waste product of digestion (haematin), varied significantly between different donors and blood storage times (Figure 3.1- Figure 3.6). In all experiments, mosquitoes obtained larger blood meals (more haematin) from feeding on blood that had been stored for 7 days than from that stored for 14 or 21 days. However although statistically significant differences were detected (facilitated by the large sample sizes), these actual magnitude of the predicted differences in blood meal sizes between blood of different storage times were relatively small (2-8%). Consequently while I can conclude that blood storage age may make some contribution to mosquito blood meal size as assayed under laboratory conditions, it is a relatively minor one.

These predicted differences in blood meal size due to blood storage age translated into differences in mosquito oviposition rate (which was highest on blood stored for 7 days), but did not have clear impacts on fecundity or survival. Blood storage age was only significantly related to mosquito fecundity in interaction with mosquito wing length (Figure 3.8), meaning that there was no blood storage age that was consistently better for fecundity than others. Furthermore, mosquito longevity was not influenced by the length of time blood had been stored prior to feeding.

In comparison to these modest effects of blood storage time on mosquito fitness, the impacts of blood donor were largely absent. Mosquito blood meal size varied between individual donors under some blood storage conditions (e.g. 7 days), but not others (blood stored for 14 and 21 days). Furthermore, neither mosquito oviposition rate, fecundity, nor post-feeding survival was significantly influenced by blood donor.

The results of these preliminary experiments suggest that fresh blood (stored for up to seven days) is the best source of blood for future mosquito fitness experiments because of its association with enhanced oviposition. Using blood from a range of different donors should not introduce a significant source of variation into mosquito fitness

measurements. However, to minimize even the minor variation in mosquito fitness that may arise from using blood of different human donors, in the rest of my PhD studies, within an experiment, different treatments were generated using blood from the same donor (manipulated to have different red cell density).

3.5.1 Additional remarks

Red blood cells cannot be stored with serum as the blood is liable to coagulate, making it unusable. Thus for the experiments described here, blood was kept refrigerated at +4°C resuspended in medium, and erythrocytes were then resuspended with a fixed volume of serum to achieve a standardized packed cell volume (PCV). As red cells are a key source of protein within the blood for mosquito reproduction, their density in blood (as indexed by PCV) is expected to influence mosquito fitness and thus was standardized here by mixing equal volumes of red cells and serum together to achieve a consistent PCV (40%). The PCV of each blood meal was recorded immediately prior to mosquito feeding (Table 3.1). While careful attempts were made to standardize the PCV of all blood treatments here, the resulting PCV of blood mixtures did vary somewhat between treatments (due to random experimental error). As can be seen in Table 3.1, the PCV of 14 and 21 day old blood for all donors was lower than that for 7-day old blood (X^2_2= 7162.1, $p < 0.001$). The cause of this variation is not known, but could have been due to changes in the preparation method of blood meals (different centrifuge speeds used to pellet the cells before resuspension in serum), or to haemolysis occurring during storage. If mosquito blood meal size and subsequent fitness is related to small differences in PCV, this experimental error may have contributed to some of the apparent treatment effects. However it would not explain the apparently smaller blood meals taken by mosquitoes feeding on 7 day old blood from donor A, compared to blood from donors B and C of the same age (section 3.4.1). Further research is required to confirm whether the moderate impacts of blood storage age reported here are independent of this variation in PCV.

	STORAGE TIME OF BLOOD		
Donor	7 day	14 day	21 day
A	42%	31%	32%
B	41%	31%	31%
C	42%	32%	33%

Table 3.1. Packed cell volumes of the blood meals used (measured by haematocrit centrifugation (section 2.3).

3.6 Conclusion

Mosquito blood meal size varied slightly between human host donors and blood storage age. Mosquitoes took the largest blood meals from blood that had been stored for the minimum amount of time (for 7 days rather than for 14 or 21 days). In contrast to mosquito blood meal size and oviposition rate, there was no difference in the longevity of mosquitoes fed blood from the different donors and from blood of different storage ages. The impacts of blood donor on blood meal size, oviposition rate, fecundity and longevity were minimal or absent.

The observations suggested that fresh blood (stored for up to seven days) was the best source of blood for future mosquito nutrition experiments. However, blood from different donors was not found to influence mosquito fecundity and survival. Therefore blood from different donors was obtained weekly for the rest of the experimental work.

Chapter 4. The impact of erythrocyte density on *Anopheles* mosquito fitness

4.1 Aims and objectives

The aim of this experiment was to investigate the effect of variation in the PCV of human blood on the fitness of the primary vector of human malaria in Africa, *Anopheles gambiae* s.s.. The key questions investigated were whether variation in the PCV of human blood across a range corresponding to normal (50% PCV) and severe anaemia (15% PCV) influenced the feeding success and subsequent fitness of this vector in terms of its blood meal size, egg production and long-term survival.

The hypothesis tested was that blood with a low PCV representative of severe anaemia had a lower fitness value to *An. gambiae* s.s than host blood of normal PCV. The results of this study will be discussed in the context of the potential epidemiological consequences of variation in host haematology, and its role in shaping the outcome of vector-parasite interactions in malaria.

4.2 Introduction

Anaemia is a serious health threat to people in developing countries (Bukar *et al.*, 2008), and in Africa is common in women and children (Bukar *et al.*, 2008). Poor nutrition, iron deficiency, and infectious diseases such as malaria and helminth infections increase the likelihood of anaemia (Taylor & Hurd, 2001; Nkuo-Akenji *et al.*, 2006). Pregnancy, and especially multiple pregnancies, can also contribute to anaemia in women. For example, 92.6% of pregnant women in Gombe, Nigeria were anaemic as a result of parasitic infections such as helminth worm infections and malaria (van den Broek *et al.*, 1998; Bukar *et al.*, 2008).

Anaemia is defined as having a packed red cell volume (PCV) of < 30%, which is equivalent to haemoglobin (Hb) levels of < 13 g/dl (Bukar *et al.*, 2008). For adult African males, the normal range of PCV is 40-50% (Hb 14-18 g/dl). For adult African females, PCV usually ranges from 30-46%, (13-15 g/dl Hb) (Fairbanks & Tefferi, 2000). PCV and Hb measurements are considered as haematological indicators for classifying the severity of anaemia, with PCVs in the range of 21-30% and Hb 9.5 -13 g/dl being considered as mild

anaemia, PCVs of 15-20% and Hb 8-9.5 g/dl as moderate anaemia, and < 15% PCV and Hb < 8 g/dl as severe anaemia (Mukiibi *et al.*, 1995). As PCV is a measure of the number of erythrocytes per unit volume of blood, and haemoglobin the amount of protein (in erythrocytes) per unit volume of blood, PCV and Hb levels are generally highly correlated (Fairbanks & Tefferi, 2000). Anaemia occurs when there is a reduction in the number of erythrocytes to plasma in whole blood, which indicates a concurrent reduction in both PCV and Hb.

Anaemia can be caused by several different physiological and pathological features such as reduced production of erythrocytes (dyserythropoiesis), deficiency in nutrients, excessive destruction of erythrocytes, hereditary diseases, excessive blood loss, for example through gastrointestinal ulcers, and infectious disease (Shulman *et al.*, 1996; Abdelrahim *et al.*, 2009; Mogensen *et al.*, 2006b; Kurtzhals *et al.*, 1999; Nsiah *et al.*, 2010; Nweneka *et al.*, 2010). While anaemia has important consequences for the health of affected people, it may also influence the resource value of their blood to mosquitoes and other haematophagous insects that feed upon them. Many insect vector-borne diseases such as malaria both cause anaemia, and occur with greatest frequency in developing countries where other causes of anaemia such malnutrition are common. Consequently insect disease vectors commonly encounter anaemic hosts, and any change in host blood quality caused by this condition that influences vector fitness could limit their transmission potential. This could arise both through direct impacts on vector fitness and ability to survive through the pathogen's extrinsic incubation period, or through indirect impacts on the reproductive success of pathogens within their vectors (e.g. due to host energetic limitation). Variation in host PCV is likely to influence the number of erythrocytes that mosquitoes obtain during a blood meal. As red blood cells are the primary resource mosquitoes use for egg production (Hurd *et al.*, 1995), a reduction in total intake would be expected to decrease their reproductive output. Blood is also an important resource for mosquito long term survival (Hurd, 2001; Maier *et al.*, 1987; Clements, 2000; Aboagye-Antwi *et al.*, 2010). As parasites such as those causing malaria require a relatively long period of development within their mosquito vectors before they can be transmitted to a new host (e.g. at least 10 days for malaria, (Baton & Ranford-Cartwright, 2005b; Beier, 1998)), any reduction in mosquito survival during this period will have a huge impact on parasite transmission potential.

Mosquitoes feeding on blood from anaemic hosts are likely to produce fewer eggs and to survive for a shorter length of time than mosquitoes feeding on a healthy person with higher levels of haemoglobin. However, it is possible that anaemia-induced reductions in PCV could have positive as well as negative impacts on malaria vectors and the parasites they transmit. This is because one aspect of mosquito feeding efficiency, the rate of blood intake, is negatively correlated with PCV. Previous studies have demonstrated that *An. stephensi* mosquitoes were able to imbibe significantly larger blood meals when the PCV of their rodent hosts decreased from normal (45-59%) to intermediate levels (43-44%) as a consequence of infection by the rodent malaria *P. yoelii nigeriensis* (Taylor & Hurd, 2001). However, when host PCV fell further to 15-35% as parasitaemia increased, mosquitoes obtained smaller blood meals than those feeding on blood of normal PCV (Taylor & Hurd, 2001). The hypothesised cause of this phenomenon is the relationship between PCV and blood viscosity, with blood of high PCV being 'thicker' and requiring more energy to imbibe (Daniel & Kingsolver, 1983). In contrast, the relatively 'thinner' consistency of low PCV blood allows mosquitoes to imbibe blood at a faster rate and thus consume a higher total volume within a fixed period of time (Shieh & Rossignol, 1992; Taylor & Hurd, 2001). However at low PCV values, the advantage of faster blood uptake may become outweighed by lower red cell density which diminishes mosquito energetic intake.

These trade-offs between the rate of blood intake and its PCV content have been hypothesized to generate a curvilinear relationship between host PCV and mosquito reproduction (Taylor & Hurd, 2001), with blood of intermediate PCV being the best resource for mosquito fitness. However in most previous investigations of this phenomenon, host anaemia has been created by malaria infections (Taylor & Hurd, 2001; Ferguson *et al.*, 2003b); thus it has not been possible to separate clearly the impacts of parasites from PCV on mosquito fitness. By directly manipulating host red cell density independently of infection, the experiments described in this chapter specifically tested the impact of PCV on the fitness and transmission potential of malaria vectors.

4.3 Methods

4.3.1 Experimental design and sample size calculation

Groups of *An. gambiae* s.s. female mosquitoes were fed on human blood representing 2 different blood PCV treatments (representing normal PCV (45-50%) as a healthy individual and low PCV (10-15%) to mimic severely anaemic patients). The experiment was repeated seven times (blocks).

Preliminary sample size calculations were conducted to estimate the number of mosquitoes needed in each experimental block in order to be able to detect a statistically significant difference in fitness traits. These calculations were performed on the basis of the mosquito fitness trait assumed to exhibit the most variable: fecundity (number of eggs laid per individual). Details of sample size calculation based on pilot data of the variability in this trait are described in appendix 8.3. On the basis of this analysis, a total of 130 female mosquitoes per experimental treatment (normal or low PCV) in each block were identified as sufficient to detect a minimum difference of 10% between blocks with 90% power.

4.3.2 Mosquito blood feeding

Mosquitoes were reared followed standard methodology as described in Chapter 2 (section 2.1). Adult females were collected and transferred into the prepared pots (approx 130 per pot) three to five days post emergence, two days before the blood feeding (section 2.4). The preparation of the blood meal followed standard methodology as described in section 2.3, and was fed to mosquitoes by membrane feeding followed established protocols (Carter *et al.*, 1993) [section 2.4]. Two membrane feeders were run concurrently, and replicated seven times. Within each independent replicate, blood from a different human host was used (manipulated to be either normal or low PCV). During each feeding trial, mosquitoes were allowed to feed from membrane feeders for 15-20 minutes.

4.3.3 Mosquito fitness parameters

The amount of blood taken by individual mosquitoes during feeding was estimated indirectly by measuring the amount of haematin excreted after blood feeding, which is

correlated with the total mass of erythrocytes imbibed (Briegel, 1980) as described (section 2.5). Mosquitoes were transferred to separate new plastic tubes for oviposition (section 2.6).

From the first day after blood feeding, all mosquitoes in tubes were examined daily to check whether they were alive, and to record their day of death. After death, the body size of each mosquito was estimated by measuring their wing-length [a standard indicator of body size] (Briegel, 1990b) as described (section 2.7). Survival of mosquitoes was monitored until natural day of death (section 2.7).

4.3.4 Statistical analysis

The impact of PCV variation on four indicators of *An. gambiae* s.s. fitness was measured: blood meal size, fecundity, oviposition rate and survival. In all analyses, blood PCV treatment was investigated as the primary effect of interest, with mosquito wing length fitted as an additional fixed explanatory variable in all cases. Furthermore, the effect of replicate (7 per treatment) was fitted as a random effect. Associations between the continuous variables of blood meal size and fecundity, and the explanatory variables of wing length and PCV were tested using the General Linear Mixed Model procedures (GLMM) in the R statistical package (lmer, R statistical software v.2.10.1/2.12.2, (Crawley M.J., 2007)). The model with the highest degree of statistical support [as evaluated on the basis of the backward elimination procedure (section 2.8.1)] was selected as the "best" model. A similar approach was adopted to test for statistical differences between the binary response variable of oviposition (yes/no). Oviposition data was evaluated by using logistic regression analysis in R (glmer, R statistical software v.2.10.1/2.12.2, (Crawley M.J., 2007)).

The impact of host PCV on mosquito survival was analyzed in two different ways. Firstly, survival analysis using the Cox proportional hazard model was used to evaluate the impact of host PCV on mosquito mortality rate from the time of feeding until their natural death. In this model, the random effect of 'replicate' was incorporated by fitting a frailty function (Hougaard, 1995). The models were built up as described in section 2.8.1. Secondly, the impact of PCV on mosquito survival up until day 14; which is the approximate length of time required for parasites to complete their extrinsic rate of

development (Ferguson & Read, 2002a; Ferguson *et al.*, 2003a; Baton & Ranford-Cartwright, 2005b) to test the influence of PCV on the ability of vectors to sustain parasite development. This 'transmission-relevant' survival parameter was analysed by testing for significant differences in the proportion of mosquitoes surviving until day 14 using the generalized linear mixed models procedure in R for binary data as described above (glmer, R statistical software v.2.10.1/2.12.2, (Crawley M.J., 2007)).

4.4 Results

4.4.1 Blood meal size

In this experiment, approximately 130 female mosquitoes from each experimental block were offered a blood meal of different PCV (50% and 15%). The total number of mosquitoes offered a blood meal in each replicate, the number that fed, and their average blood meal size (and standard error, SEM) are shown in Appendix 8.4. Mosquito blood meal size was significantly influenced both by the interaction between host PCV and their own body size (X^2_1= 13.96, P< 0.001, Figure 4.1). There was a positive relationship between blood meal size and mosquito body size for both host PCV groups (normal PCV: X^2_1= 38.18, P< 0.001; low PCV: X^2_1= 19.84, P< 0.001), however the rate of increase in blood meal size with mosquito body size was significantly greater in the normal PCV than low PCV cohort.

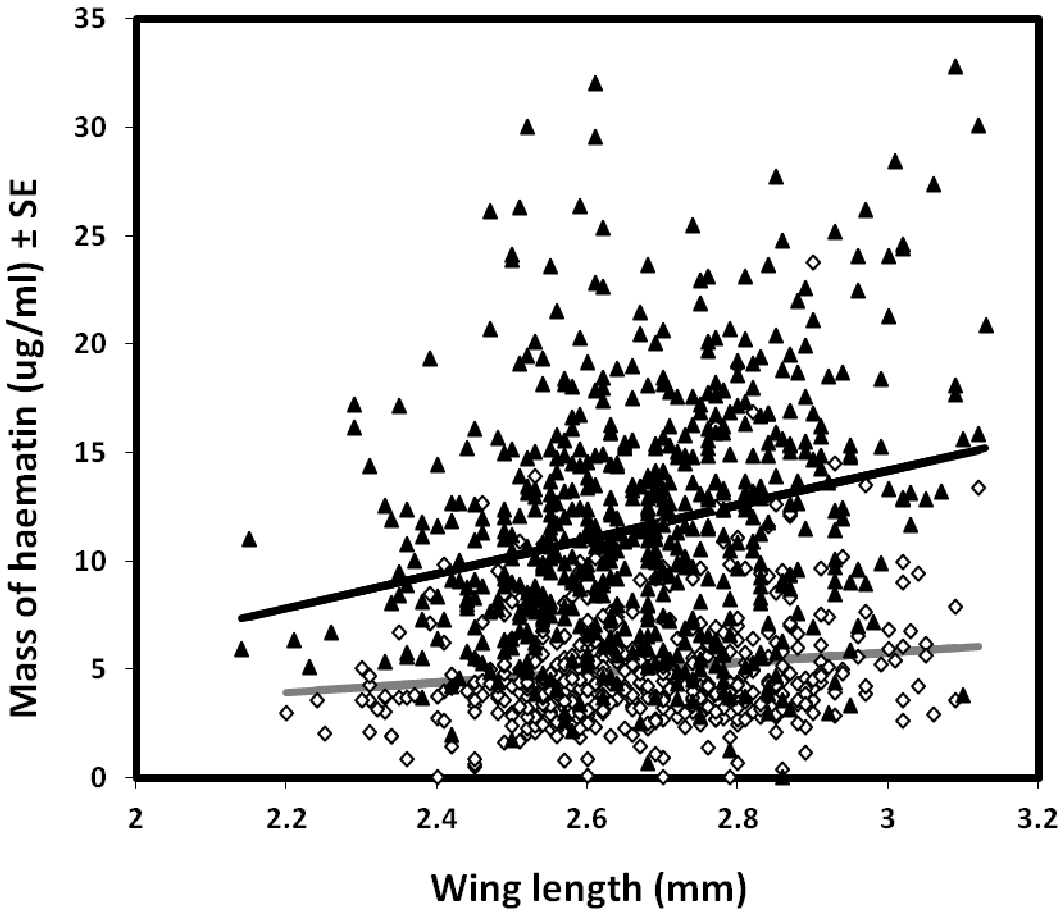

Figure 4.1. Relationship between mosquito body size, host PCV and the size of *An. gambiae* s.s. blood meals. Each point represents one mosquito. Mosquitoes fed on normal blood are shown as black triangles and those fed on blood of low PCV as white diamonds. The regression lines show statistically significant relationships (normal PCV: solid black line; low PCV: solid grey line).

4.4.2 Oviposition rate

The number of mosquitoes taking a blood meal and the numbers that subsequently laid eggs are given in Appendix 8.4. Statistical analysis indicated that although mosquitoes obtained larger meals from blood of normal than low PCV, there was no difference in mosquito oviposition rate between host blood treatments (X^2_1= 2.5, *P*=0.11, N=1047, Figure 4.2). However, mosquito oviposition rate was influenced by mosquito wing length (X^2_1= 22.45, *P*<0.001), with larger mosquitoes being more likely to lay eggs than those had smaller ones (Figure 4.3).

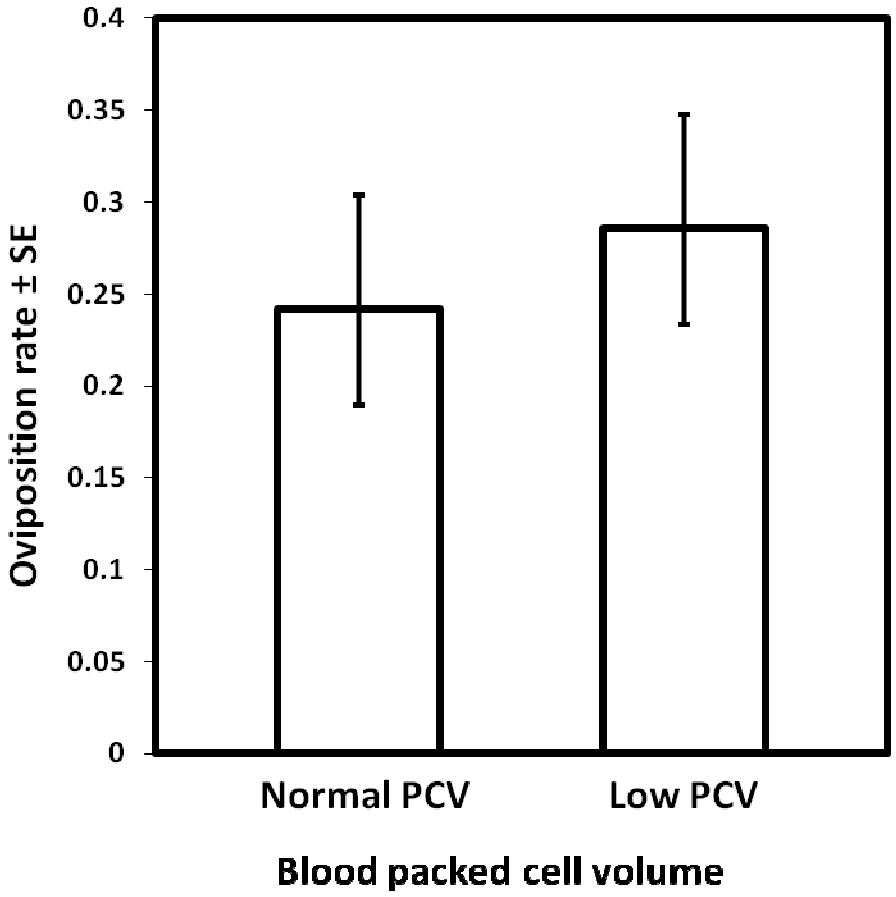

Figure 4.2. The oviposition rate of *An. gambiae* s.s. after feeding on human blood of different PCV levels. Bars show the estimated coefficient (β) of mosquito oviposition rate (proportion of mosquitoes that laid eggs) predicted by the logistic regression statistical models (N=1047, seven replicates), Error bars represent ± 1 standard error (SE).

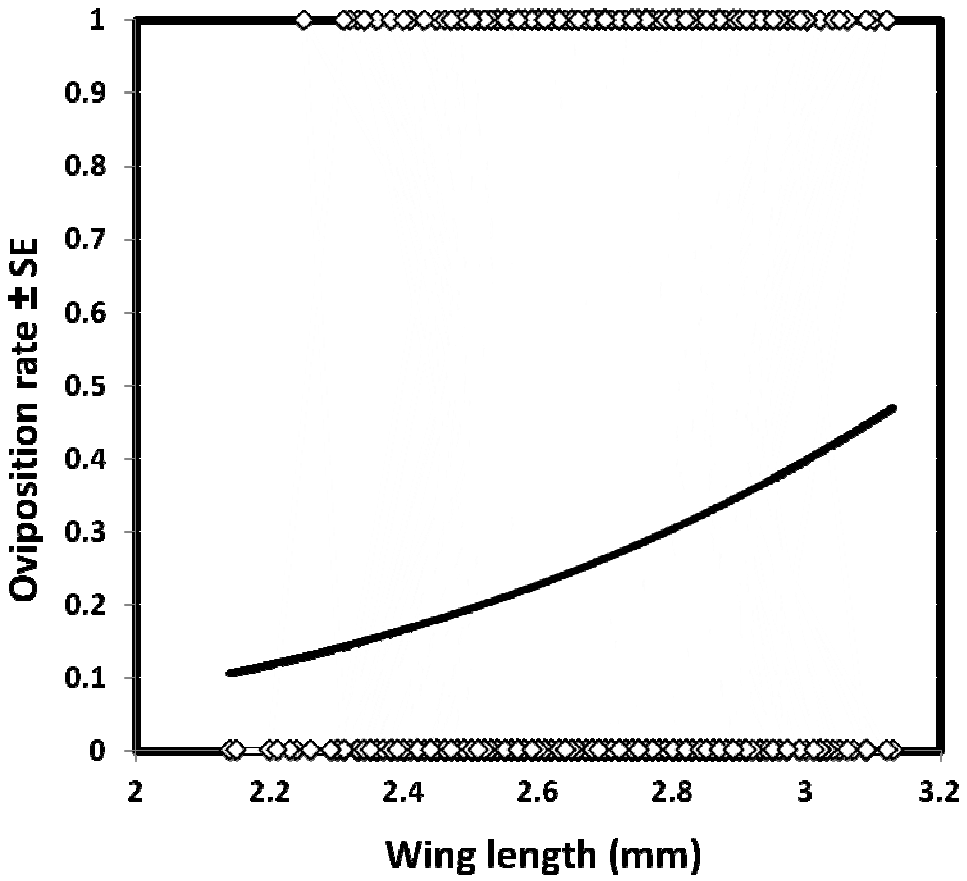

Figure 4.3. The relationship between the size of *An. gambiae* s.s. and oviposition rate. Points represent mosquitoes which laid or did not lay eggs (binary response variable). The logistic regression shows the statistically significant relationship based on the estimated parameters of the model (solid black line, N=1047).

4.4.3 Mosquito fecundity

Mosquitoes that failed to lay at least one egg were excluded from further analysis of fecundity. The mean number of eggs laid by *An. gambiae* s.s. feeding on different host blood PCVs are shown in Appendix 8.4. Mosquito fecundity was significantly related to both their body size (X^2_1= 28.50, *P*< 0.001) and the host PCV (X^2_1= 35.13, *P*< 0.001, Figure 4.4). For a given body size, mosquitoes feeding on blood of low PCV were predicted to lay approximately 15% more eggs than those who fed on normal blood as estimated by the regression analysis. There was no significant interaction between PCV and wing length (X^2_1= 0.6, *P*= 0.44).

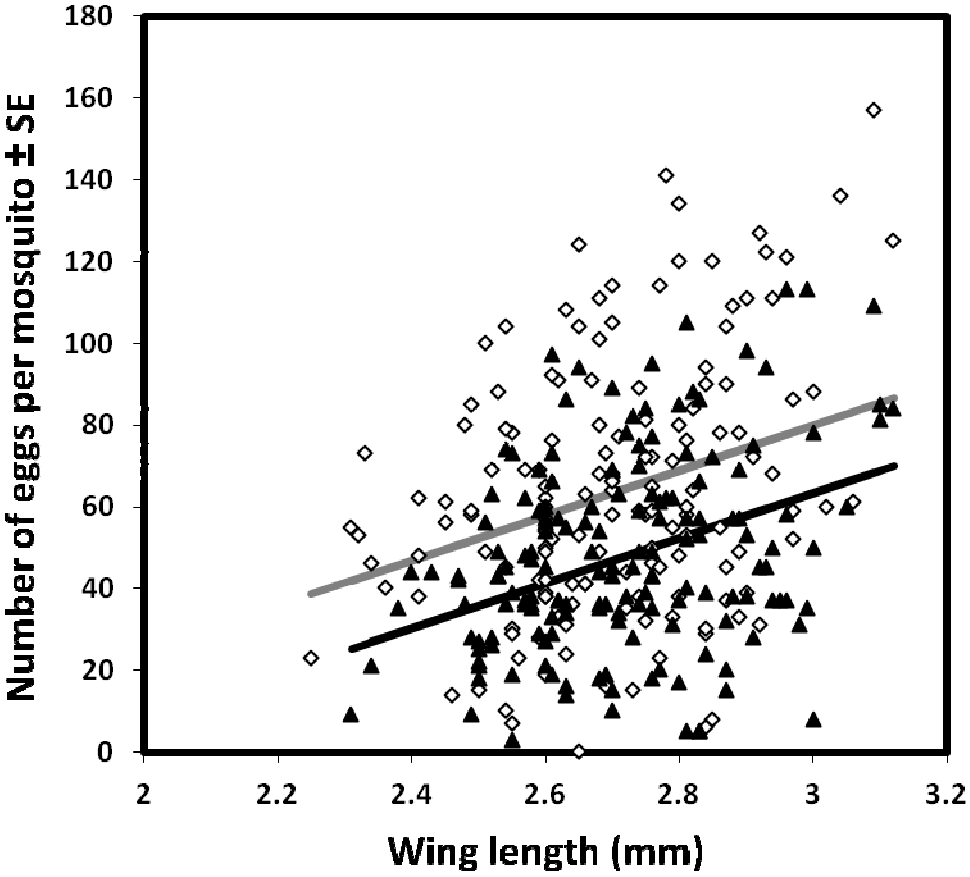

Figure 4.4. The relationship between mosquito body size, host blood PCV and the number of eggs laid by *An. gambiae* s.s. females. Mosquitoes feeding on normal PCV blood are shown as black triangles and those feeding on blood of lower PCV as white diamonds. The regression lines are as predicted by the best-fit statistical model (normal PCV blood: solid black line; low PCV blood: solid grey line).

4.4.4 Survival

The survival of all blood fed mosquitoes was monitored, irrespective of whether they did or did not lay eggs. As reproduction is known to be costly and to reduce longevity in many insects (Anderson & Roitberg, 1999; Carey *et al.*, 2008; Dao *et al.*, 2010; Stone *et al.*, 2011; Flatt, 2011; De Loof, 2011), it was first checked whether there was a significant independent impact of oviposition on *An. gambiae s.s.* survival that needed to be accounted for before testing for the impact of host PCV. The analysis revealed that mosquitoes that laid eggs had a significantly higher survival than those that did not (X^2_1= 15.80, P< 0.001, Figure 4.5). Overall the risk of mortality in mosquitoes that laid eggs was approximately 28% lower than in those that did not (Odds ratio ± SE = 0.72 ± 0.08 , 95% CI: 0.61- 0.85, N= 795, Figure 4.5).

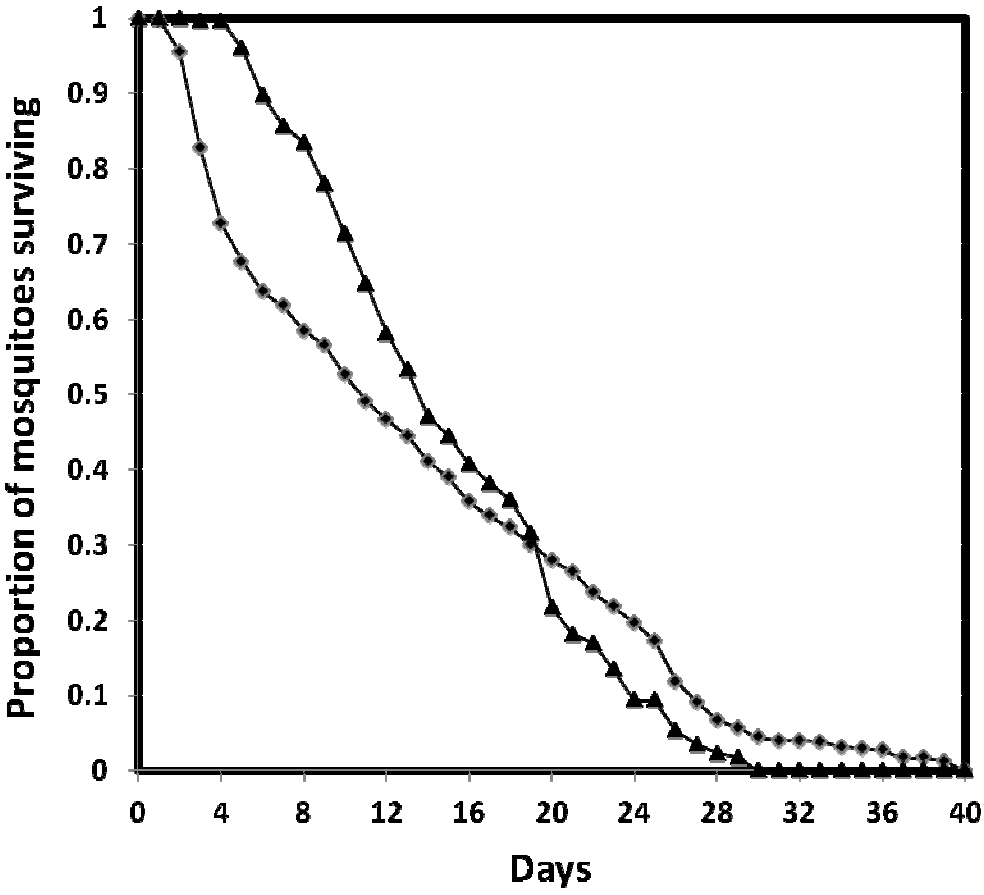

Figure 4.5. Survival of productive female mosquitoes (*An. gambiae* s.s. which laid eggs) after feeding on human blood (black triangles) and non productive female mosquitoes (*An. gambiae* s.s. which did not lay eggs, black diamonds). Each point was calculated by predicted survival of all replicates and weighting of points depending on variation in the sample size within a replicate. The output was from the model of survival curve, Cox's Proportional Hazards model (N= 795).

All subsequent analyses of the impact of PCV on mosquito longevity were thus performed with the inclusion of oviposition (yes/no) as an additional explanatory variable. In this analysis, both oviposition (laid eggs/ non-laid eggs, odds ratio of mortality ± SE= 0.70 ± 0.08, 95% CI: 0.59- 0.82, X^2_1= 14.12, *P*<0.001) and blood PCV (normal PCV/ low PCV, odds ratio of mortality ± SE= 0.75 ± 0.07, 95% CI: 0.65- 0.87, X^2_1= 10.99, *P*<0.001; Figure 4.6) had a significant impact on mosquito survival. The survival of mosquitoes in this experiment was unrelated to their body size (X^2_1= 0.42, *P*= 0.51).

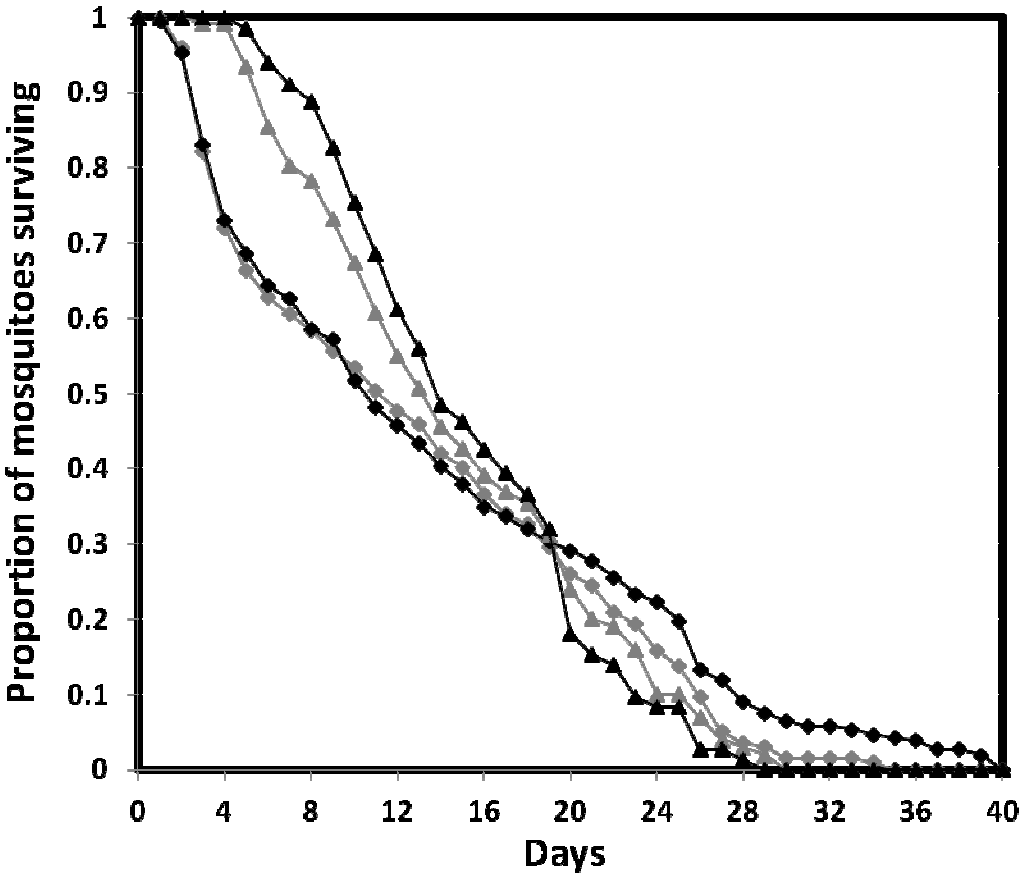

Figure 4.6. Survival of *An. gambiae* s.s. female mosquitoes that laid (triangles) and did not lay (diamonds) eggs after feeding on human blood of normal PCV (black) or low PCV (grey). Each point was calculated by predicted survival of all replicates and weighting of points depending on variation in the sample size within a replicate. The output was from the model of survival curve (N=795).

Human malaria parasites require approximately 14 days to complete their extrinsic incubation period within mosquitoes before they can be transmitted to a new host (Ross, 1898). Logistic regression analysis revealed that the survival of mosquitoes during the 14 days after blood feeding was affected by mosquito oviposition rate (X^2_1= 11.10, *P*< 0.001, Figure 4.7) but not blood PCV (X^2_1= 1.71, *P*= 0.19). The survival of mosquitoes was unrelated to their body size (X^2_1= 0.91, *P*= 0.34).

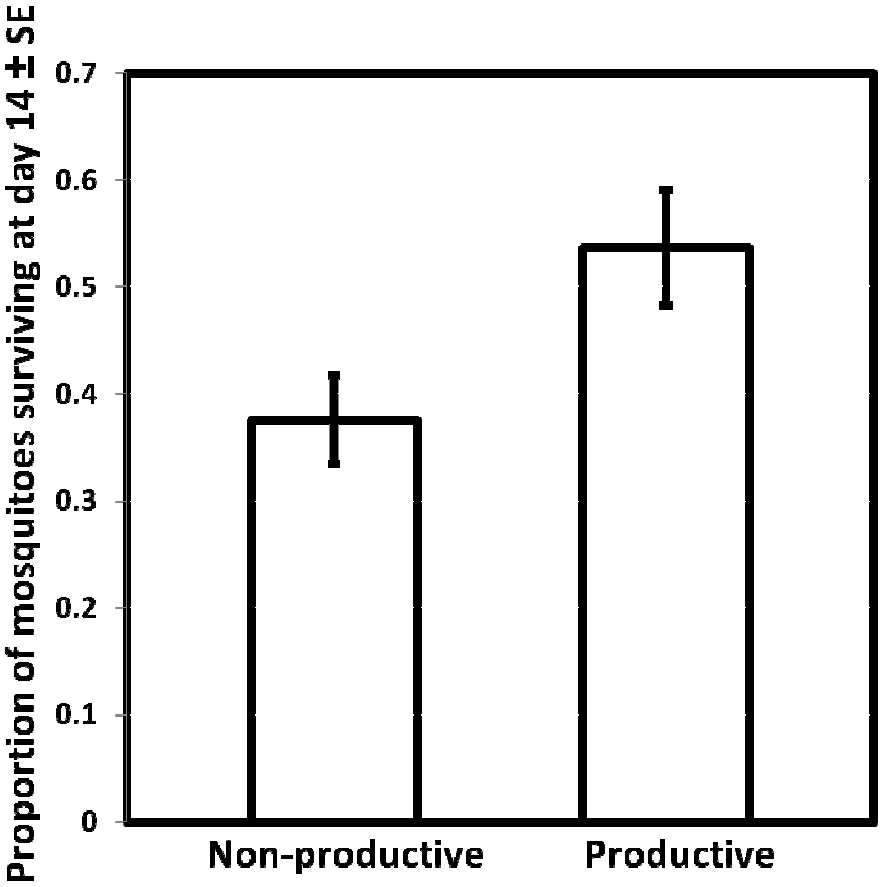

Figure 4.7. The proportion of *An. gambiae* s.s. surviving to day 14 after blood feeding, in relation to whether they did or did not lay eggs. The figures represent parameters predicted by the logistic regression statistical models. Bars show the β estimates of mosquito survival rate (proportion of mosquitoes surviving at day 14) in productive and non-productive mosquitoes and error bars represent ± standard error (SE) of the coefficient (β) that is estimated by the model.

4.5 Discussion

In this study the amount of protein that malaria vectors acquire from blood-feeding, and their resultant long term survival, was found to be positively associated with the PCV of human blood. However, contrary to initial predictions, mosquitoes feeding on blood with low PCV had the same oviposition rates as those feeding on blood of normal PCV, and also showed an increased fecundity of around 15%. This study also provides evidence of a higher survival rate in mosquitoes that laid eggs (productive) after feeding compared to those that did not. This effect was strongest in the earlier stages of mosquito lifespan (survival to day 14, equivalent to the extrinsic incubation period of malaria parasites).

The observed reduction in blood meal size in mosquitoes feeding on blood with low PCV is consistent with previous studies conducted on rodent models (Ferguson *et al.*, 2003b;

Taylor & Hurd, 2001). Specifically, anaemia in mice due to infection with *P. chabaudi* (which reduced their PCV by 20% in comparison to uninfected controls) was associated with a 25% reduction in the blood meal size of *An. stephensi* (Ferguson *et al.*, 2003b). A similar study of the rodent malaria parasite *P. yoelii nigeriensis* found that the blood meal size of *An. stephensi* fed on infected mice with low PCV (15-35%) and high parasitaemia was lower than those fed on infected mice with normal PCV (42-45%) (Taylor & Hurd, 2001). However, as host anaemia in both studies occurred as a consequence of malaria infection, the impacts of infection-induced anaemia and the parasites themselves could not be disentangled. My results on *An. gambiae* s.s. are in agreement with these studies, and demonstrate clearly that PCV variation on its own, in the absence of host infection, is responsible for reductions in mosquito blood meal size.

Not all studies, however, have shown that reductions in host PCV result in a decrease in mosquito blood meal size. A theoretical model based on the physics of capillary flow predicted that mosquitoes should have the fastest rate of erythrocyte and total protein uptake when feeding on blood with a PCV level of somewhat below normal (Daniel & Kingsolver, 1983; Shieh & Rossignol, 1992), because moderate reductions in red cell density reduce blood viscosity and thus the rate mosquitoes can imbibe blood. This advantage is predicted to be outweighed by the cost of the lower protein content (red cell density) of blood as its PCV falls (Daniel & Kingsolver, 1983; Shieh & Rossignol, 1992). In addition, when *Aedes aegypti* mosquitoes engorged completely, the overall volume of blood which they consumed per second from moderately anaemic (low PCV) rabbit blood (29%) was similar to that of normal PCV (41%) (Shieh & Rossignol, 1992). However, the reductions in PCV tested in this study was relatively moderate (PCV= 29%) (Shieh & Rossignol, 1992) compared to that evaluated here (PCV < 15%, consistent with severe anaemia in humans), confirming that the advantages of increased blood flow rate are outweighed by the cost of reduced red cell density at more extreme levels of anaemia (e.g. PCV 15%).

In contrast to impact of host PCV on blood meal size, mosquitoes fed on low PCV blood had a similar oviposition rate to those fed on normal PCV blood. This similarity was evident even after controlling for variation in mosquito wing length, which was positively correlated with oviposition rate here and elsewhere (Lyimo & Takken, 1993; Briegel, 1990a). It is unclear why extreme reductions in host PCV significantly reduce mosquito

blood meal size but not their oviposition rate, as in previous studies blood meal size has been shown to be positively correlated with oviposition (Hurd *et al.*, 1995; Hogg & Hurd, 1995a), and egg development (Hogg & Hurd, 1995b; Ahmed *et al.*, 2001; Gakhar *et al.*, 2002). Possible reasons why the blood treatment associated with the lowest blood meal size here (e.g. low PCV) had no effect on oviposition rate in the present study could be that the oviposition rate of mosquitoes fed on low PCV blood was enhanced by other chemical substrates in the host serum such as free amino acids, lipids or other chemical elements. However, currently there is no evidence that blood serum factors might be important in triggering oviposition. Regardless of the mechanism, these results suggest that blood meal size may be an unreliable indicator of the mosquito oviposition rate when females feed from hosts with blood of variable PCV.

Mosquitoes feeding on low PCV blood laid a higher number of eggs: of mosquitoes that laid at least one egg, those fed on low PCV blood laid approximately 15% more eggs than mosquitoes fed on blood of normal PCV. Additionally, fecundity was positively related to wing length as has also been shown in previous studies, *An. gambiae* s.s. fecundity was positively related to wing length (Briegel, 1990a; Reisen & Emory, 1977; Lyimo & Takken, 1993). The finding of enhanced fecundity with low PCV blood are in contrast to my initial prediction, and to previous studies, which have shown that as host PCV decreases due to malaria infection, both mosquito blood meal size and fecundity are reduced (Hogg & Hurd, 1995a; Briegel & Rezzonico, 1985a). The enhanced fecundity of mosquitoes fed on low PCV blood here cannot be due to its higher quality as indexed by protein intake from haemoglobin, as the haematin assay indicted that the blood meals had reduced protein levels. An alternative possibility apparent reproductive advantage of mosquitoes fed on low PCV blood (ovipositon and fecundity) is that they compensated for the lower quality of their blood meal by reallocating other energetic reserves (e.g. lipids or other chemical elements), acquired during larval development, to reproduction, which allowed them to lay more eggs in the short-term, but possibly at a cost of long term survival, or subsequent fecundity. Allocation theory predicts that in maximizing their fitness, organisms face a trade off between the allocation of resources to short-term versus long-term reproduction (Stearns & Stearns, 1992; Carlson & Harshman, 1999; Price, 1984). Trade-offs between survival and reproduction are predicted to be most extreme when resource availability is low (Stearns & Stearns, 1992). This theory has been investigated in the model organisms *Drosophila* and *Ceratitis capitata* in a large range of studies which

have shown that insects can increase their short-term reproduction at the expense of longer term survival when resources are low (Graves, 1993; Chapman & Partridge, 1996; Chapman *et al.*, 1998; Good & Tatar, 2001; Piper *et al.*, 2005).

Studies of other mosquitoes have shown that such life-history trade-offs can occur (Roitberg & Friend, 1992). For example, *Aedes aegypti* and *Aedes albopictus Skuse* increased their reproductive potential and basic reproductive rate in restricted dietary situations (Harrington *et al.*, 2001; Braks *et al.*, 2006). Likewise, *An. gambiae* s.s. had lower survival rate but higher life time fecundity when offered only blood and water in the absence of sugar compared to blood and sugar (Gary & Foster, 2001). Taken together, this evidence suggests that when mosquitoes (or other insects) are faced with low resources, they can shift resource allocation to short-term reproductive increases (Chapman *et al.*, 1998; Good & Tatar, 2001; Carlson & Harshman, 1999; Anderson & Roitberg, 1999; Carey *et al.*, 2008). This phenomenon may explain why mosquitoes fed on low PCV blood had higher short-term fecundity but lower long-term survival here.

Although variation in blood PCV had a significant impact on mosquito long-term survival, it did not influence *An. gambiae* s.s. throughout the minimum period required for malaria parasite development (up to 14 days post feeding). This suggests that even extreme variation in host blood PCV may have limited impact on the vectorial capacity of *An. gambiae* s.s. mosquitoes. Although mosquito survival through the parasites extrinsic development is the most crucial determinant of transmission potential, any effect of PCV on mosquito longer-term survival beyond this period could also affect transmission by reducing the number of biting opportunities mosquitoes have when infectious. Consequently, variation in host blood PCV may be expected to have a minor impacts on malaria transmission in terms of mosquito long-term survival.

The relatively modest although mostly statistically significant differences in survival associated with feeding on host blood of different PCV reported here may be an underestimate of what occurs in the wild where environmental conditions are harsher and more heterogeneous. Under standardised insectary conditions, mosquitoes are given access to glucose *ad libitum* after blood feeding. Previous studies have shown that sugar feeding enhanced mosquito survival (Nayar & Sauerman, 1975; Okech *et al.*, 2003; Impoinvil *et al.*, 2004; Manda *et al.*, 2007b; Stone *et al.*, 2011). Although there is some

evidence that mosquitoes take sugar from plant sources in the wild (Impoinvil *et al.*, 2004; Manda *et al.*, 2007b), it is unlikely to be to the same extent as in the laboratory. Thus the provision of glucose to mosquitoes in these experiments may have minimized the fitness costs of host low PCV by providing additional energetic resources to offset the poor quality of low PCV blood. Furthermore, during this experiment mosquitoes were held under standardized laboratory conditions and exposed to few of the environmental stresses that would be encountered in nature (e.g. temperature and humidity fluctuation, high energetic demand of finding an oviposition site, predators). Further field-based experiments considering the impact of human host PCV variation on mosquito fitness under natural conditions will thus be required to confirm the role of host haematological factors on mosquito fitness.

Mosquito body size has been widely demonstrated to influence blood meal size and fitness (Hurd *et al.*, 1995). In this study, it was shown that there was a positive correlation between mosquito blood meal size and wing length as expected, but relationship was different depending on blood type (normal and low PCV). Specifically, mosquitoes fed on blood of normal PCV were able to increase their blood intake at a much faster rate in relation to their body size than those fed on low PCV blood. In this latter group, the proportionate increase in blood meal size with body size was much less pronounced. This evidence may indicate that extreme variation in host haematological factors can be just as important or more than mosquito own body size in determining their blood resource intake.

4.6 Conclusions

The results in this chapter demonstrate the impact of blood meals of low PCV on the fitness of the *An. gambiae* s.s.. As expected, the size of blood meals obtained by mosquitoes feeding on blood of low PCV was significantly lower than from blood of normal PCV. However, despite the reduction in blood meal size, mosquitoes that fed on low PCV blood had a similar oviposition rate and produced more eggs (approximately 15%) than those fed on blood of normal PCV. One explanation of these results is that mosquitoes may re-allocate their energetic reserves from long-term survival to short-term fecundity when blood resources are of poor quality. Even if such a re-allocation can occur, it is not clear whether it could completely compensate for the impact of poor

blood meal quality (low PCV). For example, it is not known if the eggs laid by mosquitoes fed on low PCV blood are of the same quality as those produced from normal blood. It is possible these have been provisioned with fewer maternal reserves and will have a lower hatch rate than those produced from normal PCV hosts, but this was not tested in the experiments to date.

There are potential implications of these results for malaria transmission. Host blood PCV did not influence mosquito survival rate until day 14. Low PCV may not affect the numbers of mosquitoes reaching day 14, when they are ready to transmit, but any effect on survival after that may still have some, albeit relatively minor, influence of malaria transmission (e.g most mosquitoes would only live long enough for one transmission opportunity anyway).

Chapter 5. The impact of erythrocyte density on the infectiousness of malaria parasites to their mosquito vectors

5.1 Aims and objectives

The aim of this experiment was to investigate the effect of variation in the PCV of human blood on the fitness of the major human malaria parasite, *Plasmodium falciparum* in a major natural vector, *Anopheles gambiae* s.s. The key aims were to investigate were whether variation in the PCV of human blood across a range corresponding to normal (50% PCV) and severe anaemia (15% PCV) influenced the infectiousness of malaria parasites to mosquito vectors, and subsequent development inside mosquitoes to human-infectious transmission stages (sporozoites).

5.2 Introduction

There is a large body of literature describing the deleterious effects of malaria infection on their vertebrate hosts, with the most common impacts including cerebral malaria, severe anaemia, weight loss, increased plasma levels of histidine and histamine, respiratory distress, renal failure, acidosis, hypoglycaemia, and coma (Enwonwu *et al.*, 2000; Trampuz *et al.*, 2003). Several of these symptoms can rapidly lead to death within hours or days [reviewed in (Castelli *et al.*, 2010; Menezes *et al.*, 2012)]. Of these symptoms, anaemia is often viewed as an unavoidable by-product of malaria infection because of the parasite's obligate invasion and destruction of red blood cells [reviewed in (Castelli *et al.*, 2010)]. In malaria endemic regions, severe anaemia is common because of repeated or chronic infection by Plasmodium and other parasitic co-infections such as helminths in children (Kurtzhals *et al.*, 1999; Scolari *et al.*, 2000; Nkuo-Akenji *et al.*, 2006; Bouyou-Akotet *et al.*, 2009; Gulen *et al.*, 2011; Mboera *et al.*, 2011), multiple pregnancy in adult females (Steketee & Campbell, 2010; Akinboro *et al.*, 2010) and malnutrition (Abdelrahim *et al.*, 2009; Ribera *et al.*, 2007). The pathophysiology of anaemia due to malaria is complex (Menendez *et al.*, 2000), but the most common characteristic of this condition is a reduction in the packed cell volume (PCV) of erythrocytes and an associated decrease in blood haemoglobin concentration (Hb) (Shiff *et al.*, 1996; Menendez *et al.*, 2000).

In addition to its impact on human health, reductions in red cell density caused by malaria may also modify the likelihood of subsequent parasite transmission to mosquito vectors. Several hypotheses have been forward for how this could occur, the first being that mosquitoes acquire larger blood meals and thus have a greater probability of imbibing parasites when host red cell density is low (Taylor & Hurd, 2001; Daniel & Kingsolver, 1983; Shieh & Rossignol, 1992; Briegel, 1990b). Blood meal size determines the number of infectious malaria gametocytes that mosquitoes are likely to consume and thus may influence parasite transmission (Briegel & Rezzonico, 1985a; Briegel, 1990b; Hurd, 1990b; Hurd *et al.*, 1995). The volume of blood imbibed by Anopheline mosquitoes is influenced by the density of host red blood cells (Shieh & Rossignol, 1992; Briegel & Rezzonico, 1985a). Through a process called prediuresis, some anophelines (Vaughan *et al.*, 1991) can concentrate and retain erythrocytes in the blood meal while excreting plasma during blood feeding (Briegel & Rezzonico, 1985a). As only red blood cells are used for mosquito nutrition (Briegel & Rezzonico, 1985b; Vaughan *et al.*, 1991), this process allows mosquitoes to maximize their resource intake by increasing the volume of blood that they consume. As well as allowing the mosquito to consume a higher number of red blood cells that would be otherwise expected from their density in blood, prediuresis also increases the number of gametocytes (which infect red blood cells) consumed (Vaughan *et al.*, 1991). However, previous studies indicate that prediuresis does not allow the mosquitoes to fully compensate for the reduction in red cells in malaria infected hosts (Taylor & Hurd, 2001), suggesting that both parasite transmission and mosquito fitness may suffer a negative impact from host anaemia.

However, several other theoretical and empirical studies have indicated that host anaemia influence parasite infectiousness in other ways. For instance, hosts with low PCV may exhibit less defensive behaviour, and thus allow mosquitoes to imbibe greater numbers of red cells and thus parasites than on an otherwise healthy host (Daniel & Kingsolver, 1983). In addition, parasites in hosts with low PCV blood may have enhanced gametocytogenesis. In *P. falciparum* culture, low PCV can trigger gametocyte production (Schneweis *et al.*, 1991). In laboratory model systems, gametocyte production rates have also been shown to increase with anaemia (Paul *et al.*, 2004; Reece *et al.*, 2005). Additionally, in natural human infections, gametocyte density is often negatively correlated with blood PCV, generating a positive relationship between host PCV and their infectiousness to mosquitoes (Drakeley *et al.*, 1999; Smalley & Brown, 1981; Rosenberg *et*

al., 1984; Drakeley *et al.*, 1999; Price *et al.*, 1999; Nacher *et al.*, 2002). In the above studies, the apparent increased infectiousness of anaemic hosts is thought to be primarily due to their higher gametocyte densities. However it is possible that host PCV can have an impact on malaria parasite infectiousness independent of gametocyte density. Specifically, mosquito blood meals taken from hosts with low PCV may be substantially less viscous than those from host with normal blood. This reduced viscosity in the blood meal could increase the likelihood of sexual fertilisation in malaria parasites, by enhancing the motility of gametes and thus their efficiency to find one another in the blood meal within the crucial period before blood meal digestion. Evidence of an additional positive impact of anaemia for malaria infectiousness to mosquitoes would suggest that some level of parasite virulence (as manifested by anaemia) in vertebrate hosts can enhance transmission, a central prediction of virulence-transmission trade-off theory (Levin *et al.*, 1982; May & Anderson, 1983; Ewald, 1983; Stearn & Koella, 2008; Schwartz & Koella, 2001; Day, 2001).

In addition to the effects described above, host anaemia could have additional and as yet unconsidered impacts on malaria transmission success by enhancing parasite development throughout the sporogonic cycle. In previous studies, the impact of host anaemia on parasite infectiousness has usually been evaluated by comparing the number of parasites that successfully invade the midgut (Drakeley *et al.*, 1999; Rosenberg *et al.*, 1984). While this is a useful index of transmission, it is unknown whether the subsequent development of transmission stages within these oocysts (sporozoites) is influenced by the red cell density of the infectious blood meal. While the number of oocysts that infect a mosquito is generally related to the gametocyte density in a blood meal (Mitri *et al.*, 2009; Drakeley *et al.*, 1999), the subsequent replication of infectious-stage sporozoites within these oocysts may also depend on the amount of nutrients available to parasites within mosquitoes. Early studies of the development of *P. falciparum* in its vector *An. gambiae* found that while oocysts were of uniform size in low density infections, at higher density the size of oocysts varied widely (Sinden & Strong, 1978b). This variation was hypothesized to be the result of competition between oocysts for mosquito nutrients which are limited when parasite density is high (Sinden & Strong, 1978b). The relationship between the number of sporozoites per oocyst and the intensity of infection has been investigated by Bell and Ranford-Cartwright (manuscript in preparation) using *P. falciparum* in *An. stephensi* mosquitoes. Sporozoite numbers were the same in oocysts in

mosquitoes fed a single infected blood meal, or a second uninfected blood meal at day five after the infected blood feed, and were not influenced by the intensity of oocyst infection. However, when mosquitoes were given five blood meals (infectious blood meal plus 4 uninfected feeds), the number of sporozoites that developed in individual oocysts was substantially increased (Ranford-Cartwright and Bell, manuscript in preparation). All in all, these studies suggest that the quantity of red cells consumed by mosquitoes can influence the development of malaria parasites in mosquitoes, and thus that reductions in host red cell density due to anaemia could influence malaria transmission success by reducing the efficiency of sporozoite production in oocysts. To our knowledge, this possibility has not yet been evaluated.

To address these knowledge gaps, in this chapter experiments were conducted to compare the infectiousness of the human malaria parasite *P. falciparum* to its primary mosquito vector *An. gambiae* s.s. under conditions mimicking blood meals from hosts of normal red cell density and those seen in people with severe anaemia (haemoglobin < 10 g/dl). The in vitro experimental system used made it possible to modify the PCV of infectious host blood while standardizing the density of infectious gametocytes; uniquely allowing specific evaluation of the impact of red cell density on the infectiousness of malaria parasites to mosquitoes and their subsequent replication to host transmissible stages.

5.3 Methods

5.3.1 Experimental design

Human blood and serum were mixed together to create two different PCV treatments: one representing blood of normal PCV (50%), and another blood of a person with low PCV characteristic of severe anaemia (15%) (section 2.3). The same volume of *P. falciparum* gametocyte-infected blood (thus containing a similar inoculum of gametocytes) was added to tubes which contained either normal or low PCV blood (same volume in each tube). This meant that blood of different PCV were seeded with a similar number of gametocytes, and thus that the density of gametocytes was similar in both blood treatments even though their PCV varied. These blood treatments were fed to groups of female *An. gambiae s*.s. (section 2.4) to assess the impact of host PCV on: (1) parasite infection rate (as indicated by the proportion of mosquitoes infected with oocysts), (2)

parasite intensity (number of oocysts per infected mosquito) and (3) development of human infectious transmission stages (total number of sporozoite genomes per infected mosquito). The experiment was replicated seven times.

5.3.2 Parasite culture

5.3.2.1 Asexual stages

A clone of the human malaria parasite *P. falciparum,* denoted 3D7, which is known to produce gametocytes infectious to mosquitoes (Walliker *et al.*, 1987), was used for all experimental infections. Parasites were cultured *in vitro* according to standard protocols (Trager & Jensen, 1976), at 5% haematocrit in complete RPMI1640 medium with 10% human serum under a gas environment of 1% O_2, 3% CO_2 and balance N_2. The culture medium was changed every day, and thin blood smears taken and stained with Giemsa to assess the parasitaemia every two days. When the parasitaemia reached ~6% (approximately 2 days after initiating the culture), cultures were diluted with a freshly made 5% haematocrit mixture of blood/complete medium.

5.3.2.2 Parasite gametocyte culture

New parasite (*P. falciparum*) culture samples were thawed every two or three months to ensure good gametocyte producing ability of the cultures and infectiousness of gametocytes (Carter *et al.*, 1993; Ponnudurai *et al.*, 1982). Gametocyte cultures were set up at 0.5-0.7% parasitaemia, 6% haematocrit in complete RPMI medium according to standard procedures (Carter *et al.*, 1993). For each infectious feed, a mixture of gametocytes from a 14 day old and a 17 day old gametocyte cultures was used (Carter *et al.*, 1993). Gametocytaemia was assessed two days before the infectious blood meal and also checked on the day of the feed by examination of Giemsa-stained blood smears.

5.3.3 Preparing the infectious feed

On the day of the experimental infections, blood representing two different PCV levels was supplemented with *P. falciparum* gametocytes obtained from *in vitro* cultures. The gametocyte inoculum was prepared by removing medium from the culture flasks, and mixing the remaining red cells from D14 and D17 cultures together before transferring into a 10ml centrifuge tube. The mixed culture was centrifuged for 5 minutes at 1500 x *g*.

The supernatant was then removed and a volume of serum equal to size of the pellet was added and mixed well. An equal volume (~ 400 µl) of this gametocyte mixture was added to 1.5 ml of each of the blood treatments of different PCV. By adding a fixed volume of well-mixed gametocyte culture to a fixed volume of each blood treatment, it was ensured that parasite density (no. gameocytes per ml of blood) was constant in both PCV groups. Within an experimental replicate, the volume of parasite culture added to low and normal PCV was identical. However, the exact volume (~ 400 µl) of infectious material added to blood treatments varied slightly between replicates in accordance to the density of gametocytes in cultures on the day of experiments, with the aim being to achieve a baseline of approximately 0.7% gametocytaemia in the normal PCV group which is known to generate good infection prevalence (>50%) in *An. gambiae* s.s. mosquitoes in the University of Glasgow insectary (Ranford-Cartwright, pers. comm.).

5.3.4 Mosquito Membrane feeding

Mosquito membrane feeding was conducted as previously described in chapter 2 (section 2.4). Separate groups of mosquitoes were transferred into holding labelled pots, and were offered either infected blood of low or normal PCV blood from a member feeder. For each replicate, two pots of mosquitoes were fed on the low and two on the high PCV blood.

5.3.5 Estimating parasite infection in mosquitoes

Blood-fed mosquitoes were maintained under secure insectary conditions (section 2.4) for 10 days before being killed and dissected to assess if they were infected (as judged by the presence of oocysts on their midgut). Before dissection, mosquitoes were exposed to fatal doses of chloroform in batches of 4, and then were submerged in 70% ethanol before transfer to a 5ml tube containing sterile phosphate buffered saline (PBS) and kept on ice until dissection. Individual dissections were performed with mounted 26 gauge syringe needles under a dissecting microscope in sterile PBS on a clean microscope slide. Midguts were removed from each mosquito and placed under a coverslip. The number of oocysts on the gut was then counted under a 40X objective (400X magnification) on a compound microscope. After counting the oocysts, whole dissected midguts on which oocysts were observed were placed into individual 1.5ml micro-centrifuge tubes containing 500µl of lysis buffer [L15] (Charge Switch®gDNA micro tissue kit, Invitrogen,

UK, CS 11203). Proteinase K (20mg/ml in 50 mM Tris-HCl, pH 8.5, 5 mM $CaCl_2$, 50% glycerol) was then added to each micro-centrifuge tube to obtain a final concentration of 1mg/ml. Midguts were incubated overnight at 55^{o}C, and then stored at -80^{o}C until DNA extraction (Ranfordcartwright *et al.*, 1991).

5.3.6 DNA extraction from infected mosquito midguts

DNA was extracted using a standard protocol (Charge Switch®gDNA micro tissue kit, Invitrogen, UK, CS 11203) following the manufacturer's protocols. Briefly, 5µl of RNase A (5mg/ml in 10mM Tris-HCl, pH8.5, 10mM EDTA) was added to each DNA sample and incubated at room temperature for 5 minutes. Next 200 µl of purification buffer (N5) was added to each sample tube and mixed by gentle pippetting. Forty microlitres of ChargeSwitch® magnetic beads from the kit were then added to each tube and gently mixed before incubating for 1 minute at room temperature. During this step, tubes were placed in the MagnaRack® which pulls the magnetic beads towards a magnet thus permitting removal of the supernatant but not the parasite DNA which is bound to the magnetic beads. These beads were then washed twice with 1 ml of wash buffer (W12 solution from ChargeSwitch® gDNA micro tissue kit) and the supernatant discarded. Finally, the DNA was eluted from the beads with 150µl of elution buffer (E5, 10mM Tris-HCl, pH 8.5), and the purified DNA samples stored at -80 ^{o}C.

5.3.7 Quantitative real-time polymerase chain reaction (qPCR) assay

Quantitative real- time PCR (qPCR) has been carried out for the determination of the number of malaria parasites circulating in peripheral blood for use in clinical trials (Schoone *et al.*, 2000; Cheesman *et al.*, 2003; Farcas *et al.*, 2004; Malhotra *et al.*, 2005), and recently for estimation of parasite replication rate in mosquitoes (Bell & Ranford-Cartwright, 2004; Bell & Ranford-Cartwright, 2002). The qPCR method allows accurate quantification of the numbers of parasites present within individual oocysts or mosquito midguts, and can be taken as the number of sporozoites developing with them (Bell & Ranford-Cartwright, 2004). Quantitative real time PCR assays amplifying the Pfg377 gene were performed to measure the amount of DNA (by implication, the number of parasite genomes) present within midgut samples. Reactions were performed on a Roche Light Cycler using the non-specific dsDNA-binding fluorescent molecule SYBR Green I as detection system for quantification.

5.3.7.1 Creation of known concentration of DNA standards

DNA standards containing known numbers of *P. falciparum* parasites were generated from asexual cultures for use in the quantitative PCR (qPCR) analysis of genome numbers (following Bell and Ranford-Cartwright, 2004). Asexual cultures of parasite clone 3D7 were synchronised using the Plasmion-based technique (Lelievre *et al.*, 2005; Ranford-Cartwright *et al.*, 2010) to provide a culture consisting of only ring-stages, with each ring-stage parasite being a single infection per red blood cell. The total number of parasites (genomes) present per microlitre of culture was calculated from (i) parasitaemia which was estimated from Giemsa-stained slides, and (ii) the associated red cell density of cultures as estimated from haemocytometer slide counts made of the culture. To estimate the total number of parasites per µl of culture, values of parasitemia were multiplied by red blood cell density of cultures. Separate 100 µl samples containing 1.25, 2.5, 5, 10, 100, 1000, 1 x 10^4, and 1 x 10^5 parasites per microlitre of culture were prepared by dilution in uninfected blood at 5% haematocrit. DNA was then extracted as described below.

Each 100 µl parasite sample yielded 100 µl of DNA in Elution Buffer, so the parasite concentrations after DNA extraction were 1.25, 2.5, 5, 10, 100, 1000, 1 x 10^4, and 1 x 10^5 parasites per microlitre of DNA. DNA was extracted from each 100ul sample of synchronized culture using the ChargeSwitch gDNA 100 µl blood kit (Invitrogen, UK, CS11010), following the same protocol as described for infected mosquito midguts (section 5.3.6), with minor differences in incubation time and handling as described in the ChargeSwitch gDNA 100 µl blood kit protocol (Invitrogen, UK, CS11010).

5.3.7.2 Quantitative PCR

The quantitative reactions were prepared in Lightcycler glass capillary reaction vessels in a final volume of 20µl, containing 600nM of each primer (377F: 5' ACTCCAGAAGAAGAAGAGCAAGC 3' and 377R: 5' TTCATCAGTAAAAAAAGAATCGTCATC 3'), 3mM magnesium chloride, and 1 x Roche Light Cycler Fast Start DNA Master SYBR Green I hot start reaction mix containing with 4µl of sample template DNA. The cycling profile comprised an initial denaturation (hot start) of 95^{o}C for 600 s and then 35 amplification cycles of denaturation 95^{o}C for 30 s, annealing 55^{o}C for 20s and extension 65^{o}C for 30s.

Each sample was run in duplicate within a single run. Each assay included five DNA standards (section 5.3.7.1) of 500, 1000, 2500, 5000, and 10000 parasites (per reaction), run in duplicate. Light Cycler data were analysed using the Fit Points method to obtain a (mean) genome number for each midgut by comparison with the standard curve drawn from the DNA standards (Bell & Ranford-Cartwright, 2004). Under these conditions the assay amplified only the 408 bp target amplicon with a characteristic melting temperature of 80.7^{o}C. There was no amplification of human or mosquito DNA, or primer-dimer formation (data not shown). DNA extracted from uninfected mosquito midguts was used as a negative control.

5.3.7.3 Production of a standard curve

Standard curves were generated for every quantitative real time PCR assay run, using the prepared samples of suitable concentrations. Curves were generated using five standard samples, run in duplicate, as presented in Figure 5.1. There is a linear correlation between the number of parasite genomes (amount of DNA) and the point in the amplification cycle at which the reaction enters the log phase, a point known as Ct (Roche Molecular Biochemicals, 1999). The final result is a linear graph (Figure 5.1), in which optimum efficiency of amplification is indicated by a slope of -3.321 (Roche Molecular Biochemicals, 1999).

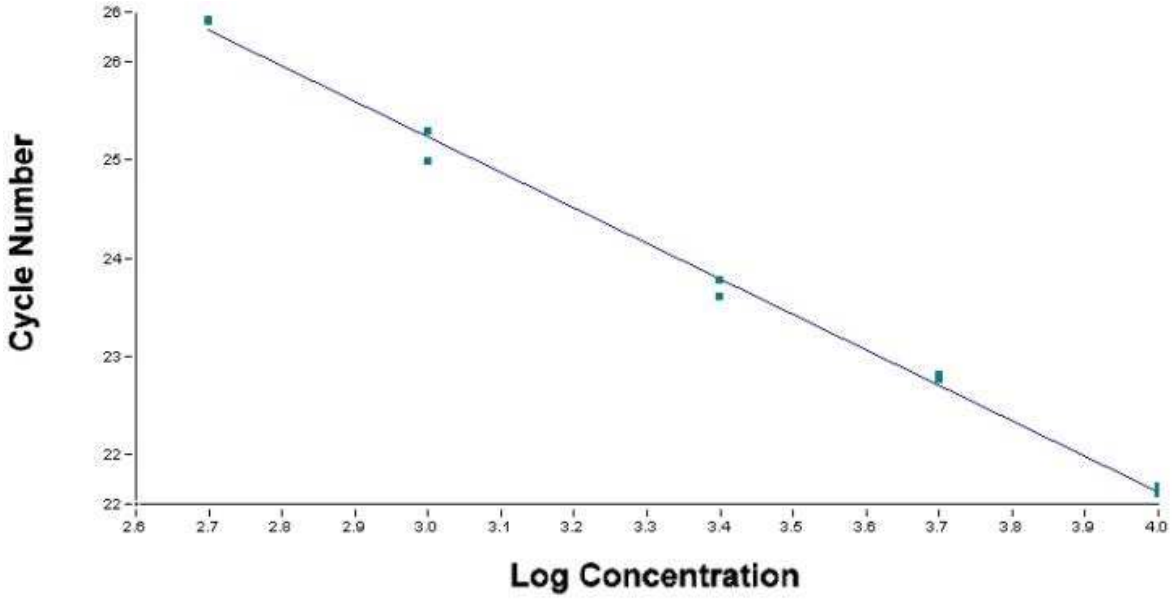

Figure 5.1. Example of a standard curve using five DNA standards containing 500, 1000, 2500, 5000 and 10000 parasites per reaction. Slope=-3.304, r^2=0.996.

5.3.7.4 Melting curve analysis

At the end of every qPCR run a melting curve analysis was carried out to confirm the presence of a single PCR product. After the last cycle of amplication, the reaction vessels are cooled to a temperature where all PCR products will be present as dsDNA. The temperature is then increased in small steps, with fluorescence readings at each step, until the PCR product denatures into ssDNA at the melting temperature (Tm) of the PCR product, at which point fluorescence will fall. A graph is plotted of the first negative derivative (-d(F1)/dT) which reveals peaks corresponding with the Tm of PCR products. The presence of a single peak indicates a single PCR product, whereas multiple peaks indicate spurious PCR product or primer-dimer formation. The Tm step consisted of a temperature rise from 65°C to 95°C, in 0.1°C /sec steps, and the expected Tm for the Pfg377 PCR product is 80.7 (Figure 5.2).

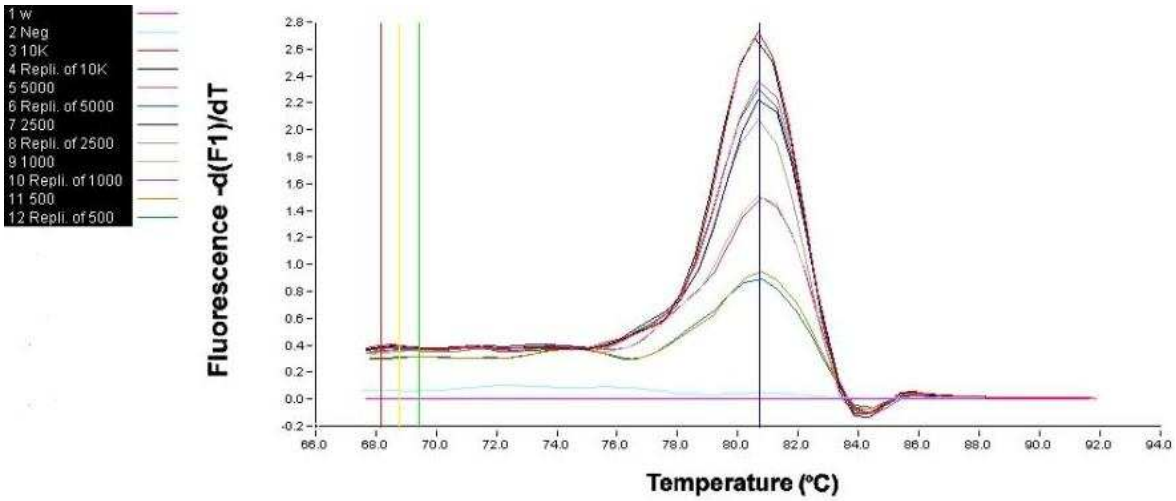

Figure 5.2. Example of a melting curve analysis from the qPCR assay carried out on standard samples (run in duplicate), and including a negative control. All PCR products have the same Tm of 80.7°C (dark blue line).

5.3.8 Statistical analysis

In this study the impact of host PCV variation on 3 indicators of *P. falciparum* infectiousness and development were measured: parasite infection prevalence (percentage of mosquitoes infected with oocysts), intensity of infection (number of oocysts per midgut) and number of parasite (sporozoite) genomes per midgut. In all these analyses, blood packed cell volume (PCV) was investigated as the primary main effect of interest. Mosquito body size was also fitted as an additional fixed explanatory variable in all cases. In addition, the effect of replicate was fit as a random effect in all analyses except for those based on the negative binomial distribution (oocyst and sporozoite number) in which it was treated as a fixed effect (as explained in 2.8).

Statistical differences between the binary response variable of infection rate (oocysts present or absent) were investigated by using the General Linear Mixed Model procedure using the binomial distribution in R (glmer, lme4 package, R statistical software version 2.12.2,(Crawley, 2007)). A maximal model was built that included all fixed effects and their interactions plus the random effect of experimental replicate. Backward elimination was used to sequentially remove non-significant variables to obtain the minimal statistically significant model (Crawley M.J., 2007; Burnham & Anderson, 1998; Burnham *et al.*, 2011). Variation in the number of oocysts and sporozoites per midgut were tested using the negative binomial model in the R statistical software (glm.nb, nlme package, R

statistical software version 2.12.2,(Crawley, 2007). For analysis of oocyst number, mosquito wing length and replicate were fitted as additional explanatory variables in a maximal model, with non-significant terms sequentially removed to obtain the minimally significant statistical model. For analysis of the total number of sporozoite genomes per midgut, the additional explanatory variable of observed oocyst intensity was also included. Oocyst intensity was reclassified into a two-level categorical variable of low (< 10 oocysts) and high intensity (≥ 10 oocysts per midgut). This categorization was justified on the basis of natural break points in the distribution of oocysts within infected mosquitoes in these experiments, and also the biological significance of this category, with the low intensity group (<10 oocysts per midgut) being representative of typical oocyst infection intensities observed in the field (Billingsley *et al.*, 1994; Harris *et al.*, 2012; Hogg *et al.*, 1995; Lyimo & Koella, 1992).

5.4 Results

Of the seven replicates conducted in this experiment, only three resulted in oocyst infections in mosquitoes. Consequently, only data from these three replicates were included in the analysis. A summary of infection rate data is shown in Table 5.1.

Replicate	Blood Packed Cell Volume (PCV)	No.dissected mosquitoes	No. infected mosquitoes	Infection rate (%)	Median oocyst intensity in infected mosquitoes
1	Normal (50%)	46	9	19.6 ± 0.04	3
	Low (15%)	50	23	46.0 ± 0.04	5
2	Normal (50%)	35	11	30.6 ± 0.05	3
	Low (15%)	34	11	31.4 ± 0.05	7
3	Normal (50%)	24	11	48.0 ± 0.06	16
	Low (15%)	25	19	86.0 ± 0.07	5

Table 5.1. Parasite infection results for mosquitoes offered gametocyte-infected normal blood (PCV= 50%) and low PCV blood (PCV= 15%).

5.4.1 Mosquito infection prevalence

Mosquitoes that fed on infected blood at low PCV were significantly more likely to become infected with oocysts than those fed on infected blood at normal PCV (X^2_1= 9.99, *P*= 0.001, N= 212, Figure 5.3). There was no statistically significant effect of mosquito

body size (X^2_1= 0.80, *P*= 0.37, N= 212) or its interaction with blood PCV (X^2_1= 2.18, *P*= 0.14, N= 212) on mosquito infection prevalence.

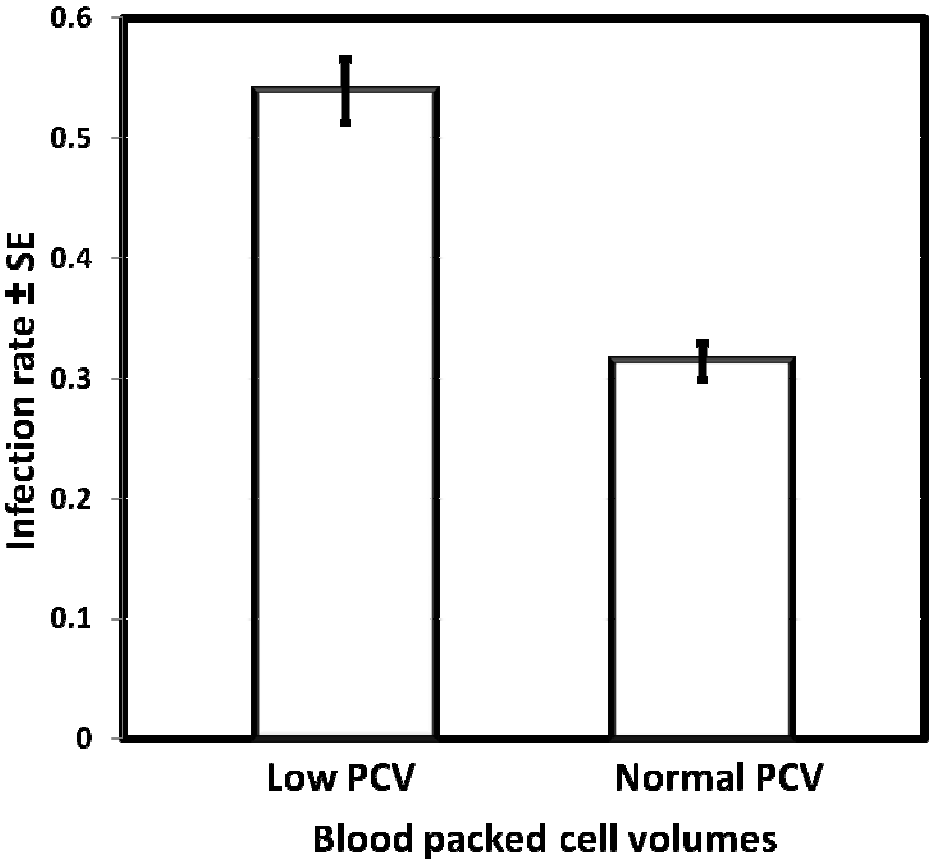

Figure 5.3. The effect of blood PCV [low:15%, and normal:50%] on oocyst infection prevalence in *An. gambiae* s.s. mosquitoes. The blocks show the estimated proportion of mosquitoes that became infected with *P falciparum* oocysts and the error bars are standard errors (N=212).

5.4.2 Malaria parasite intensity (number of oocysts per midgut)

Neither blood PCV (X^2_1= 1.28, *P*= 0.26), mosquito wing length (X^2_1= 0.05, *P*= 0.82), experimental replicate (X^2_2= 0.31, *P*=0.86) or the interaction between wing length and PCV (X^2_1= 4.01, *P*= 0.051) had a significant association with the oocyst intensity in infected *An. gambiae* mosquitoes (Figure 5.4). This analysis was carried out on mosquitoes that were observed to have at least one oocyst (zeroes excluded).

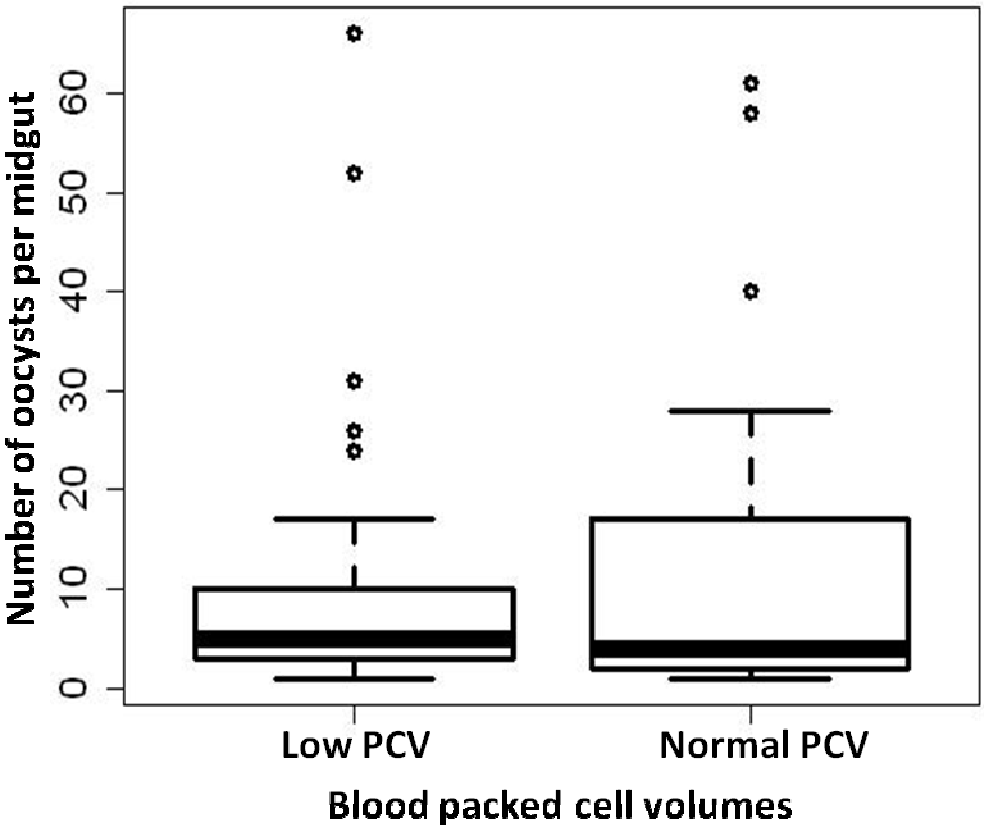

Figure 5.4. Distribution of the number *of P. falciparum* oocysts in *An. gambiae* s.s. given an infectious bloodmeal at low (15%) and normal (50%) PCV, pooled across 3 replicates. Thick black bars indicate median oocyst number per midgut, rectangles represent the interquartile range (IQR) of each PCV levels from the first quartile (the 25th percentile) to the third quartile (the 75th percentile). The whiskers extend out to the smallest value within 1.5 times the IQR from the first quartile and values larger than 1.5 times IQR from the third quartile. Circles show the values which are larger than the whiskers (outliers).

5.4.3 Total number of sporozoite genomes in mosquitoes

Neither mosquito body size (X^2_1= 1.97, *P*= 0.16) nor experimental replicate (X^2_2= 1.69, *P*=0.42) were significant predictors of the total number of sporozoites estimated to have developed in mosquitoes by day 10 post infection. There was some evidence that the PCV of the blood meal and the intensity of oocyst infection had an interactive effect on total sporozoite loads (PCV × oocyst intensity: X^2_1= 5.28, *P*=0.02). Consequently the impact of PCV was evaluated in the low and high oocyst intensity groups separately.

In mosquitoes infected with low oocyst intensities (less than ten oocysts per midgut), PCV had no significant effect on total sporozoite load (X^2_1= 1.32, *P*= 0.25). Similarly within mosquitoes who became infected with high oocyst intensities; there was no significant

difference in sporozoite load between PCV treatments (X^2_1= 0.06, *P*= 0.79). However, there was tendency for mosquitoes with a low oocyst intensity to have a higher number of genomes in the normal PCV group, whereas the opposite trend was apparent in the high oocyst intensity infected mosquitoes (Figure 5.5). This trend could have generated the borderline statistical interaction between oocyst intensity (low and high) and PCV in this analysis. However, given that the number of mosquitoes in the "high oocyst" groups was generally small (9-12 mosquitoes), further investigation is required to confirm whether host blood PCV really does influence the developmental success of parasite genome numbers in oocysts.

Separate analyses were conducted in the low and high PCV groups to determine the respective impact of oocyst density on the number of genomes (representing sporozoites) within them. In the low PCV group, mosquitoes in the high oocyst intensity group developed a significantly more sporozoites than those in the low oocyst intensity group (X^2_1= 6.75, *P*< 0.009, Figure 5.5), but this was not apparent in mosquitoes fed on gametocyte infected blood at normal PCV (X^2_1= 1.28, *P*= 0.26).

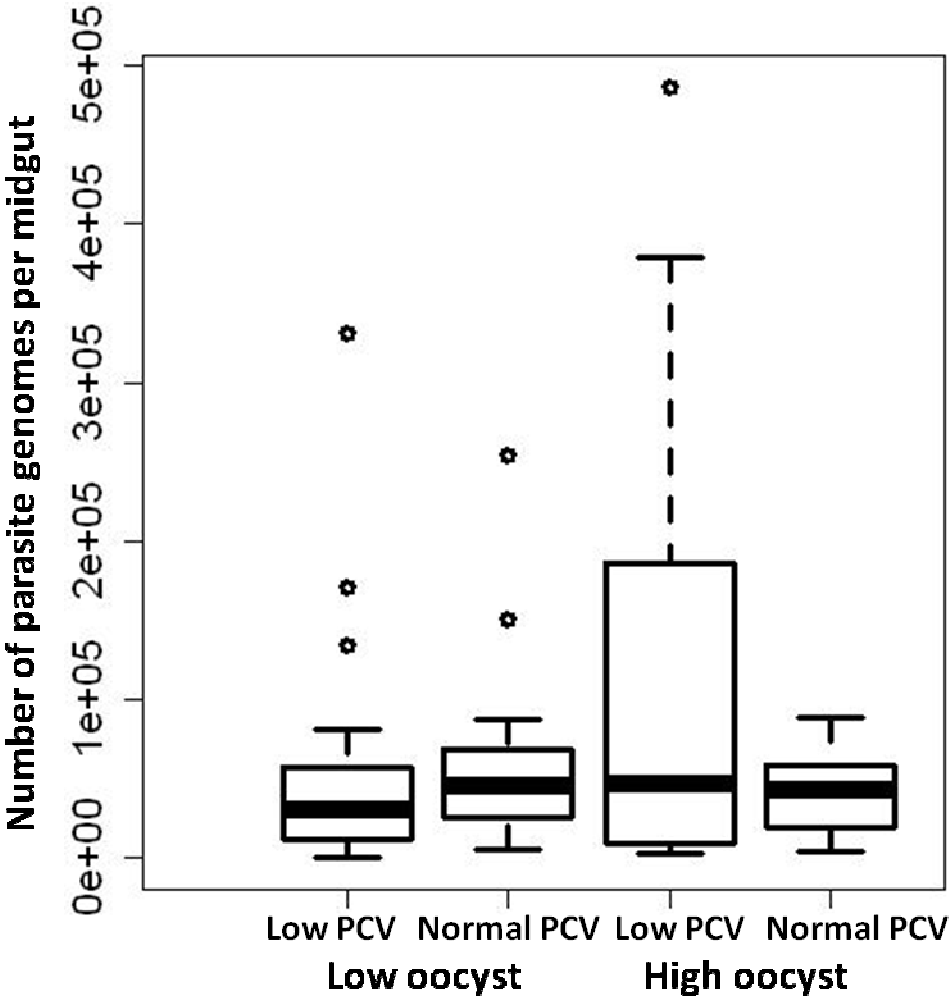

Figure 5.5. Distribution of the number *of P. falciparum* sporozoites (genomes) estimated to have developed in *An. gambiae* s.s. . given an infectious bloodmeal at low (15%) and normal (50%) PCV, with respect to their oocyst intensity (low= < 10 and high= ≥ 10 oocysts per midgut), pooled across 3 replicates. Heavy bars indicate medians, rectangles represent the interquartile range (IQR) of each PCV levels (range from first quartile (the 25th percentile) to the third quartile (the 75th percentile)). The whiskers extend out to the smallest value within 1.5 times the IQR from the first quartile and values larger than 1.5 times IQR from the third quartile. Circles show the values which are larger than the whiskers (outliers).

5.5 Discussion

In this study, the impact of human blood PCV on the infectiousness of the malaria parasite *P. falciparum* to the mosquito vector was investigated. While previous studies have tested for correlations between host factors such as PCV and parasite infectiousness in natural infections (Drakeley *et al.*, 1999; Gouagna *et al.*, 2004b; Taylor & Read, 1997; Okech *et al.*, 2004a; Nacher *et al.*, 2002; Price *et al.*, 1999; Paul *et al.*, 2004), this study is unique in that the membrane feeding assay used allowed independent estimation of the impact of host PCV while controlling for underlying variation in parasite (gametocyte) density.

Using this system, it was found that *An. gambiae* s.s. mosquitoes were substantially more likely to become infected after feeding on infected blood of low PCV rather than normal PCV. However, there was no evidence that blood PCV influenced either oocyst intensity, or the total number of sporozoites that had developed in mosquito vectors 10 days after the infectious blood meal.

The finding that low blood PCV can promote parasite infectiousness, independently of gametocyte density, could be explained by the reduced viscosity of low PCV blood making it easier for mosquitoes to obtain large blood meals, and thus ingest a greater number of gametocytes (Daniel & Kingsolver, 1983; Shieh & Rossignol, 1992; Drakeley *et al.*, 1999; Gupta *et al.*, 1994; Taylor & Hurd, 2001; Dawes *et al.*, 2009). However this does not seem to be the case as mosquitoes obtained substantially smaller blood meals from low rather than normal PCV blood (results presented in Chapter 6). Blood meal size was estimated in terms of the mass of haematin ingested (Briegel & Rezzonico, 1985a; Vaughan *et al.*, 1991), and not directly as the volume of blood consumed, and so it is possible that the volume of infected blood consumed, and thus the number of gametocytes ingested, was underestimated. It remains unclear how this could create a bias that would cause a blood meal size advantage associated with low PCV blood (necessary to explain these results) to be estimated as a blood meal size cost. Unfortunately all currently available methods for estimating the volume of blood imbibed by mosquitoes (Briegel *et al.*, 1979) require killing them shortly after feeding, and thus would not be appropriate for subsequent monitoring of their malaria infection rate.

An additional possibility to explain the higher infection prevalence seen at low PCV is that male gametes are better able to move through a lower viscosity blood meal (in blood meals of low PCV) to find and fertilise a female gamete. It is not currently possible to measure fertilisation efficiency as there are no markers to distinguish fertilised zygotes from unfertilised female gametes, so these possibilities cannot be investigated further yet. Similarly, ookinetes once formed may be better able to migrate through a low viscosity blood meal, and thus there could be a higher rate of midgut invasion and oocyst establishment in mosquitoes fed lower PCV blood meals.

Although blood PCV was found to have a positive impact on oocyst prevalence, there was no effect on the number of oocysts in infected mosqutoes (oocyst intensity). Sporogony,

as measured by total number of parasite genomes (sporozoites) in oocyst-infected mosquitoes, was also not affected by the PCV of the blood meal, although there was a non-significant tendency for mosquitoes with a low oocyst intensity to have higher genome numbers when infected with a normal PCV blood meal rather than a low PCV meal. There is currently a gap in our knowledge about the effect of mosquito diet on the replication rate of parasites within oocysts. A second (non-infectious) blood meal during the period of oocyst development has been shown to speed up oocyst maturation times (Beier *et al.*, 1989), and to increase the number of sporozoites in the salivary glands (Rosenberg & Rungsiwongse, 1991), but this effect was thought to be due to a synchronising of oocyst development, rather than by enhancing numbers of sporozoites per oocyst (Ponnudurai *et al.*, 1990; Rosenberg & Rungsiwongse, 1991). Oocyst intensity has been reported to correlate positively with sporozoite numbers (Gamage-Mendis *et al.*, 1993; Vaughan *et al.*, 1992), although this was not observed in this study.

Overall, these results imply that host anaemia can enhance the infectiousness of gametocytes to mosquitoes (with higher oocyst infection prevalence for same gametocyte density), but does not impose a cost on the subsequent development of parasites to transmission stages (sporozoites) within mosquitoes. Equal numbers of sporozoites are predicted from infections arising from blood of low and normal PCV. In combination, these results suggest that a reduction in host red cell density can provide a significant, independent increase in malaria transmission. This effect, coupled with the previously known correlation with increased gametocyte densities (Schneweis *et al.*, 1991; Price *et al.*, 1999; Nacher *et al.*, 2002; Drakeley *et al.*, 1999) suggests that parasites that can induce anaemia may have an evolutionary advantage (Frank, 1996; Ewald, 1994; Day, 2002b; Day, 2001; Paul *et al.*, 2004; Stearn & Koella, 2008).

It is unclear whether similar effects of PCV on parasite infectiousness would be observed under natural field settings in which mosquitoes would have multiple opportunities to blood feed before or after infection (e.g. every 2-4 days, (Gillies, 1953; Clements, 2000)). There is some evidence from laboratory infections that the susceptibility of *An. gambiae* s.s. to *P. falciparum* is decreased if mosquitoes had taken uninfected blood meals before the infectious meal (Vaughan *et al.*, 1994; Gass, 1977). This was hypothesized to be due to accelerated digestion of blood meals in mosquitoes that had previously fed, which limits the time window for ookinetes to successfully transit through the midgut wall and

develop into oocysts (Vaughan *et al.*, 1994; Gass, 1977). However, transmission from naturally infected people was enhanced (higher oocyst numbers) in *An. gambiae* s.s. that had been fed two rather than one uninfected blood meal before the gametocyte-containing meal (Okech *et al.*, 2004a); suggesting that increased nutrients from blood may promote rather than decrease vectorial capacity. Consequently, although blood PCV had no impact on the number of oocysts or sporozoites in the experiments in this chapter, it may do under more natural conditions where repeated exposure to low PCV blood (anaemic individuals) over multiple blood meals could put a greater constraint on mosquito resources. Further experiments, ideally under more natural conditions, are needed to evaluate whether host blood PCV could have more long-term effects on sporogony than could be detected here.

5.6 Conclusions

Taken in combination, results from these experiments suggest that conditions such as anaemia which reduce the red cell density of human blood may provide malaria parasites with a transmission advantage by enhancing their infectiousness to mosquito vectors (higher oocyst prevalence). The number of sporozoites that develop in *An. gambiae s.s*. with low oocyst intensities (typical of those occurring the field) was found to be high – for example, median genome load for low oocyst intensity group = 34631). Other studies have reported 1000-10000 sporozoites per single oocyst (Pringle, 1965; Rosenberg & Rungsiwongse, 1991)). These numbers much higher than the number of sporozoites thought to be inoculated by a single mosquito bite (~<25, (Beier *et al.*, 1990b)), and so it is unlikely that transmission is limited by oocyst intensity or parasite replication within oocysts. Thus a lack of effect of blood PCV on oocyst or sporozoite load is less important, whereas an enhancement of oocyst prevalence would be expected to provide an overall transmission advantage to malaria parasites.

Evidence of an additional positive impact of anaemia for malaria infectiousness to mosquitoes independent of gametocyte density would suggest that some level of parasite virulence in vertebrate hosts can enhance transmission, a central prediction of virulence-transmission trade-off theory (Levin *et al.*, 1982; May & Anderson, 1983; Ewald, 1983; Stearn & Koella, 2008; Schwartz & Koella, 2001; Day, 2001; Cotter *et al.*, 2011). However, the variety of haematological profiles in tropical anaemia, the multiple blood feeding

behaviour of vectors in nature, the amino acid content and other biochemical factors of anaemic host blood, plus changes in host-vector behaviour, could have adverse effects on transmission which are not documented here. An understanding of the importance of additional factors is required before conclusions about the benefits of low PCV in natural settings can be drawn.

These observations suggest that changes in human haematological factors could influence malaria epidemiology in natural transmission settings. Numerous public health measures currently being implemented in malaria-endemic countries will directly influence the prevalence of anaemia in human populations, by improving nutrition, maternal health, and reducing exposure to parasitic infections including malaria and helminthic infections. While further investigation of the complexities of vectorial competence under natural settings is needed to confirm the potential epidemiological relevance of these results, it is tentatively hypothesized that such widespread public health campaigns might provide indirect benefits to malaria control by decreasing the average infectiousness of the human population. Detailed investigation of the per capita infectiousness of malaria parasites in response to changes in the prevalence of anaemia at the community level would be required to confirm this.

Chapter 6. The influence of erythrocyte density on the virulence of malaria parasites in the mosquito vector

6.1 Aim and objectives

The work in this chapter addresses the following three questions:

(1) Is the human malaria parasite (*P. falciparum*) virulent to its natural African mosquito vector *An. gambiae* s.s.?

(2) Is malaria parasite virulence to mosquitoes dependent on the PCV of the infectious blood meal? Specifically is 'virulence' more likely to be observed and/or of greater magnitude when the infectious blood meal is of poor nutritional quality as characterized by lower red blood cell density?

(3) Can parasite or blood PCV-associated reductions in mosquito fitness be attributed to reductions in their key energetic reserves (sugars, glycogen, and lipids)?

To summarise, any deleterious effects of malaria parasites on their mosquito vectors (as expressed by the magnitude of fitness reduction relative to mosquitoes fed on uninfected blood) are predicted to increase in mosquitoes infected from blood of low PCV because it will provide fewer energetic substrates (lipids and carbohydrates as obtained from red blood cells), and thus reduce the ability of the mosquito to compensate for additional energetic demands imposed by the parasite.

6.2 Introduction

In the past 20 years evolutionary biologists have become increasingly interested in predicting the evolution of pathogen virulence and its implications for human and animal health (Stearns & Stearns, 1992; Schmid- Hempel, 2011; Stearn & Koella, 2008). A central question of this research has been to answer why pathogens vary in their virulence, with some causing almost no harm and others killing a large proportion of their hosts. This variation has been hypothesized to arise from trade-offs between virulence and

transmission success in different pathogens (Stearn & Koella, 2008; Ewald, 1994; Read *et al.*, 1999; Frank, 1996). Transmission success is the critical determinant of pathogen fitness, and it has been proposed that the 'optimal' level of virulence is the one that maximizes transmission success (Frank, 1996). In cases where transmission is directly related to pathogen replication rate, evolution may favour pathogens that are highly virulent (Frank, 1996; Schmid- Hempel, 2011). In contrast high virulence could be detrimental to pathogen fitness if it causes most hosts to die before pathogens can be successfully transmitted. Thus understanding the consequences of pathogen infection to host fitness is crucial to prediction of the evolution of virulence (Stearns & Stearns, 1992; Read, 1994; May & Anderson, 1983; Read *et al.*, 1999; Frank, 1996; Schmid- Hempel, 2011).

This trade-off theory has been used predict why virulence may vary between different classes of pathogens and hosts (Frank, 1996; Ewald, 1994; Schmid- Hempel, 2011). A notable example is pathogens who rely on insect vectors to transmit them between (usually vertebrate) hosts. In these diseases it has been predicted that pathogens should be less virulent in their vectors than in their vertebrate hosts (Ewald, 1994). This is because insect vectors are vital to transmission, and usually short-lived in nature. Thus even minor reductions in their survival (e.g. virulence) could significantly reduce the number of pathogen transmission opportunities (Ewald, 1994; Frank, 1996; Day, 2001; Day, 2002a; Day, 2002b). As many pathogens also replicate in their insect vectors, it is possible that some degree of virulence in vectors may arise as an unavoidable consequence of infection. However, the general prediction is that pathogen virulence should be lower or negligible in the vectors they rely upon for transmission than in their primary vertebrate hosts (Frank, 1996; Ewald, 1994; Ewald, 1983).

Pathogen virulence in insect vectors could be manifested in at least two ways: a reduction in vector fecundity and/or survival following infection. This phenomenon has been studied most widely in malaria parasites and their mosquito vectors. Several studies on a range of laboratory model systems and in field systems have tested for an impact of malaria parasites on mosquito fitness (Aboagye-Antwi *et al.*, 2010; Koella & Agnew, 1999; Ferguson *et al.*, 2003b; Ferguson & Read, 2002a; Agnew & Koella, 1999; Hurd, 1990a; Koella *et al.*, 1998; Ferguson & Read, 2002b; Taylor & Read, 1997; Hogg *et al.*, 1997; Ahmed *et al.*, 2001; Hogg & Hurd, 1997; Hurd *et al.*, 1995; Hogg *et al.*, 1995; Hurd *et al.*,

2001b; Hurd *et al.*, 2001a; Hurd, 1990b; Dawes *et al.*, 2009). With respect to mosquito survival, results have been generally mixed with some studies finding substantial reductions associated with malaria infection (Gad *et al.*, 1979; Klein *et al.*, 1982; Maier *et al.*, 1987; Lines *et al.*, 1991; Lyimo & Koella, 1992; Hurd *et al.*, 1995; Klein *et al.*, 1986; Ferguson & Read, 2002b; Dawes *et al.*, 2009; Aboagye-Antwi *et al.*, 2010; Manda *et al.*, 2007b; Okech *et al.*, 2003; Rui-de Xue *et al.*, 2008) and others no effect at all (Boyd, 1940; Chege & Beier, 1990; de Buck, 1936; Gamage-Mendis *et al.*, 1993; Hogg & Hurd, 1997; Robert *et al.*, 1990). Studies of naturally-infected mosquitoes under field conditions have also yielded mixed results, with some suggesting the potential for malaria infection to reduce mosquito survival (Anderson & Roitberg, 1999; Anderson *et al.*, 1999a; Lyimo & Koella, 1992; Chege & Beier, 1990; Aboagye-Antwi *et al.*, 2010) and others not (Lines *et al.*, 1991). At least one explanation for these mixed findings could be their study design, with one meta-analysis finding that studies working on unnatural vector-parasite combinations in the laboratory are much more likely to find infection-related survival reductions than those working on natural systems that co-exist in the wild (Ferguson & Read, 2002b; Ferguson *et al.*, 2003a).

In contrast to effects on vector survival, malaria parasites have been found to have a much more consistent impact on mosquito fecundity. Reduction in the fecundity of mosquito vectors following malaria parasite infection has been widely reported in both laboratory model systems and natural vector-parasite combinations (Hurd, 2001; Ferguson *et al.*, 2003b; Hurd *et al.*, 1995; Hogg *et al.*, 1995; Hogg & Hurd, 1995a; Gray & Bradley, 2006). Nevertheless, a few field-based studies reported that malaria parasites had no harmful effect on mosquito fecundity (Hogg & Hurd, 1997; Yaro *et al.*, 2012). One explanation for why infection-associated reductions in vector fecundity appear more common than survival is that this is the result of an adaptive strategy by the parasite to minimize its impact on the aspects of vector fitness that are most crucial for its development (e.g. survival) at the expense of those that have no direct impact on transmission (e.g. fecundity) (Anderson & Roitberg, 1999; Dao *et al.*, 2010; Stone *et al.*, 2011; Schwartz & Koella, 2001; De Loof, 2011). Alternatively fecundity reduction may be an adaptive strategy of mosquitoes that allows them to tolerate infection by reducing investment in their fecundity. Disentangling these two hypotheses is challenging, but regardless of the mechanism behind them, these fecundity reductions suggest malaria parasites can be virulent to their mosquito vectors.

Reviewing the vector-parasite literature, there is the potential for an alternative outcomes such as that the infection could enhance vector fitness (Ryan & Kohler, 2010; Frank, 1996; Ewald, 1994). For example, a parasite that increased the survival of its vector, such as that reported in male tsetse fly infected with *Trypanosoma vivax*, would have a transmission advantage (Makumi & Moloo, 1991). Some vectors/invertebrates may bring forward their reproduction, or increase their investment in reproduction at a younger age than those who are uninfected, as a strategy to ensure reproduction before (potentially) being killed by the infection. For example, microsporidian infections of *Corophium* resulted in higher fertility of the female amphipods (Mautner *et al.*, 2007). There is also some evidence that exposure to malaria parasites could have a positive impact on mosquito vector reproduction. One field study found that *An. gambiae* s.s. mosquitoes exposed to *P. falciparum* gametocytes in the blood of naturally infected people were 6-times more likely to produce eggs after feeding than those fed on uninfected blood (Ferguson *et al.*, 2005). However in this study, none of the mosquitoes exposed to gametocytes in blood became infected, so it is unclear whether this response was a true impact of parasitism or just exposure. Thus there is evidence that the effects of host exposure to and/or infection by parasites, including malaria parasites, are not always negative in term of host reproduction.

While variation in the impact of malaria parasites on mosquito vector fitness may at least in part be explained by the co-evolutionary history of the species under study, this alone may not account for the wide variety of outcomes both within and between systems. Another as yet untested possibility is that the impact of parasites on their vectors is mediated by the quality of vertebrate host resources that accompany the infectious blood meal, and if they are sufficient to help offset any resource losses due to infection. If this is the case, parasite virulence in vectors may be determined by the three-way interaction between host, vector, and parasite factors; and could vary widely in response to host condition. The most critical vertebrate host resource for malaria parasites and their mosquito vectors is blood, and specifically red blood cells (Taylor & Hurd, 2001; Rowe *et al.*, 2009; Gass, 1977; Athreya & Coriell, 1967; Lyimo & Ferguson, 2009). Malaria vectors require protein from blood haemoglobin to initiate oogenesis (Briegel, 1990a; Briegel, 1990b), and several studies have shown that mosquito egg production increases with the concentration of red cells imbibed (Briegel & Rezzonico, 1985a; Ferguson *et al.*, 2003b; Taylor & Hurd, 2001). Furthermore, resources from blood meals can also be used to

enhance mosquito survival (Nayar & Sauerman, 1975; Rui-de Xue *et al.*, 2008; Lyimo *et al.*, 2012). Furthermore, malaria parasites require energetic resources from red blood cells to fuel their development in both vertebrate hosts (Bruce *et al.*, 1990; Bouyou-Akotet *et al.*, 2009; Janse & Waters, 2004a; Sinden, 1983; Lobo & Kumar, 1998; Vaughan *et al.*, 2009) and mosquito vectors (Menard *et al.*, 1997; Vlachou *et al.*, 2006; Billker *et al.*, 1998; Beier, 1998; Ichimori, 1989; Drakeley *et al.*, 1999; Baton & Ranford-Cartwright, 2005b). Oocysts developing in mosquito midguts take up a significant amount of lipid from mosquitoes (Atella *et al.*, 2006; Atella *et al.*, 2009). Energy reserves in malaria parasite-infected mosquitoes could thus be reduced either due to competition between *Plasmodium* and *Anopheles* (Maier *et al.*, 1987), or the requirement for extra host resources to compensate for parasite-induced damage such as tissue repair and /or costly immune responses. If this is the case, parasite virulence to mosquito vectors may be predicted to be greatest when the red cell density of the infectious blood meal is lowest.

As has been discussed previously (Chapters 4,5), there is substantial variation in the red blood cell density of humans under natural malaria transmission settings (Bouyou-Akotet *et al.*, 2009; Mogensen *et al.*, 2006a; Kurtzhals *et al.*, 1999; Abdelrahim *et al.*, 2009; Green *et al.*, 2011). This generally arises from reductions in red cell density due to anaemia, which are measured in terms of Packed Cell Volume (PCV). Anaemia is a common health problem in sub-Saharan African (Kurtzhals *et al.*, 1999; Bukar *et al.*, 2008; Bouyou-Akotet *et al.*, 2009; Green *et al.*, 2011), especially in children and pregnant women in the malaria endemic areas. For instance, the prevalence of severe anaemia in Zambia among children (6- 59 month) was around 69% over a 2 year period of study [2008-2010] (Shulman *et al.*, 1996; Kurtzhals *et al.*, 1999; Eisele *et al.*, 2011; Abdelrahim *et al.*, 2009). Not only does malaria occur in regions of Africa where anaemia is common due to other health problems (malnutrition, helminthic infections), it is also the primary cause of this condition in many settings. This is because malaria parasites infect the red blood cells of their vertebrate hosts and consume their haemoglobin resources (Sherman, 1998; Danquah *et al.*, 2010; Pagola *et al.*, 2000). During the parasite's schizogonic cycle, red blood cells are depleted as parasites sequentially invade, replicate, and then burst out of these host resources (Pagola *et al.*, 2000; Janse & Waters, 2004b; Baton & Ranford-Cartwright, 2005b). In addition to direct losses of RBCs due to parasite invasion, hosts often respond to malaria infection by phagocytises (Buffet *et al.*, 2011). Through this combination of direct parasite consumption and host responses, malaria infection can

cause reductions in red blood cell density that range from moderate (9.7 g/dl) to severe (6.9 g/dl) in natural infections (Bouyou-Akotet *et al.*, 2009); which correspond to full range of medically-significant anaemia as classified by the WHO (severe anaemia: Hb <5 g/dl, moderate: 5 < Hb < 8 g/dl, and mild: 8 ≤ Hb < 11 g/dl).

Most previous studies have investigated the potential consequences of PCV reduction to malaria transmission in terms of its influence on the probability that mosquitoes will become infected [Chapter 5 in this thesis, and (Taylor & Hurd, 2001; Drakeley *et al.*, 1999; Drakeley *et al.*, 2006; Schneweis *et al.*, 1991)]. What is unknown, however, is whether PCV reduction could have additional indirect impacts on malaria transmission by influencing malaria parasite virulence to mosquitoes and thus their probability of surviving through the parasite's extrinsic incubation period. Furthermore, previous investigation of the impact of host red cell variation on the fitness of *Anopheles* vectors and their susceptibility to malaria parasites have generally been conducted by allowing mosquitoes to feed on naturally or experimentally-infected hosts with variable levels of anaemia (Briegel & Rezzonico, 1985a; Vaughan *et al.*, 1991; Taylor & Hurd, 2001; Drakeley *et al.*, 1999; Schneweis *et al.*, 1991). These studies have shown that blood meals from infected, anaemic hosts are often associated with reduced vector fecundity. However, as discussed in chapter 5, host PCV and malaria parasite density are often correlated in natural infections (Drakeley *et al.*, 1999; White & Ho, 1992; Rosenberg *et al.*, 1984; Rosenberg & Koontz, 1984; Drakeley *et al.*, 1999; Gouagna *et al.*, 2004a; Schneweis *et al.*, 1991), making it difficult to disentangle the impacts of low host PCV from high gametocyte density on these reductions in vector fitness.

In *P. falciparum* infections in humans, only a small proportion of asexual parasites (1.8-18.2%) go on to develop into transmission stage gametocytes (Talman *et al.*, 2004; Carter & Miller, 1979) in each cycle. In rodent malarias, gametocytes generally emerge after the peak of asexual parasitaemia, when host red cell density is beginning to recover. The timing of gametocyte emergence and density also varies and seems to be influenced by host age, anaemia, drug treatment and immunity (Taylor & Read, 1997; Babiker *et al.*, 2008; Buckling & Read, 2001). For human infections, gametocyte rates in adults are not correlated with asexual parasitaemia (Drakeley *et al.*, 2006). Gametocyte rates also increase during the wet season compared to the dry season (Abdel-Wahab *et al.*, 2002), and carriage rates are higher in younger children rather than older children and adults,

but this could be correlated with lower asexual parasite densities due to increased immunity (Price *et al.*, 1999; Stepniewska *et al.*, 2008; Ouedraogo *et al.*, 2010). However, there is also evidence that submicroscopical gametocyte rates increase with age for *P. falciparum*, it is more difficult to distinguish that there is a "peak" of parasitaemia in human malaria compare to rodent malaria species (Talman *et al.*, 2004).

The aim of this study was to test whether the virulence of the human malaria parasite *P. falciparum* in its primary mosquito vector, *An. gambiae* s.s. is dependent upon the red cell density of the blood meal in which they are transmitted. Particular investigation was made of variation in the red cell density of blood meals representative of those likely to arise from severe (e.g. 'severe anaemia', < 15% PCV) and asymptomatic malaria infection (no reduction in PCV). My goal was to test the prediction that any virulence expressed by malaria parasites in their mosquito vectors, as exhibited by a reduction in their fecundity and/or survival, will be of higher magnitude in infections from low rather than normal PCV blood. This prediction is based on the hypothesis that mosquitoes infected from low PCV blood will have fewer nutritional resources with which to offset any losses due to parasite development. It is possible that neither *P. falciparum* infection (Robert *et al.*, 1990; Chege & Beier, 1990), nor anaemia on their own have any significant impact on mosquito fitness, but that these two factors combine to have a large negative impact on malaria vector fitness and associated transmission success.

In addition to measuring impacts on mosquito fitness, the abundance of the key energetic resources that mosquitoes require for survival and maintenance (glucose, glycogen and lipids (Rivero & Ferguson, 2003; Foster, 1995; Schiefer *et al.*, 1977; Oliveira *et al.*, 2011)) were also compared following blood meals of different PCV and malaria infection status. Mosquitoes use sugars as a ready-to-use energy source for maintenance (Clements, 1992; Schiefer *et al.*, 1977), glycogen reserves for flight and mating activity (Nijhout, 1994; Clements, 2000; Stone *et al.*, 2011) and lipids to fuel resting metabolism, long range flight, immune responses and long-term survival (Nayar & Vanhande, 1971; Stone *et al.*, 2011; Kaufmann & Briegel, 2004; Atella *et al.*, 2009). Comparisons of energetic reserves made it possible to assess whether variation in mosquito fitness could be associated with reductions of particular energetic reserves following blood meals of different PCV and malaria infection status. In combination these results will allow evaluation of the

hypothesis that parasite virulence in their mosquito vectors is not an inevitable outcome of infection but determined by host factors.

6.3 Methods

6.3.1 Experimental design

Groups of *An. gambiae* s.s. female mosquitoes were fed on human blood representing four different experimental treatments, consisting of mosquitoes fed on blood of normal or low PCV blood, and within each of these, one sub group received uninfected control blood, and the other blood containing infectious malaria parasites (*P. falciparum* gametocytes). In this chapter, the interaction of low blood cell density (low PCV) and parasite infection on mosquito fitness was investigated.

6.3.2 Human blood and serum

Human blood of different PCVs was prepared as described previously (see Chapter 2).

6.3.3 Parasite asexual culture

Parasite asexual culture followed standard protocols as described in Chapter 5 (section 5.3.2.1).

6.3.4 Parasite gametocyte culture

Gametocyte culture followed standard protocols as described in Chapter 5 (section 5.3.2.2).

6.3.5 Preparing the infectious feed

In all experiments, both infected and uninfected blood samples of different packed cell volumes were prepared by diluting red cells in appropriate volumes of heat-inactivated human serum as described in chapter 5 (section 5.3.3).

For the groups of mosquitoes to be fed gametocyte-infected blood, blood of the two different PCVs categories was supplemented with malaria gametocytes, as described (section 5.3.3). Blood samples were prepared to ensure that the gametocyte density of

all infected treatments (no. gametocytes / ml of blood) remained constant, even though the red cell density of blood varied. The experimental infection system used here makes it possible to vary the PCV of a blood meal while maintaining a fixed gametocyte density, permitting investigation of the independent contribution of host PCV to mosquito vector infectivity and infectiousness.

6.3.6 Mosquito fitness measurements

Blood meal size was estimated on the basis of the amount of haematin excreted by mosquitoes in the first 3 days after feeding (details presented in chapter 2 (section 2.5) and (Briegel, 1980)). After this time, mosquitoes were transferred into new tubes bearing the same ID number and containing approximately 1 cm of water to allow for oviposition (as described in section 2.6). Mosquito oviposition rate was estimated as the proportion that laid eggs from day four until day ten after the feed (day of dissection). For mosquitoes that laid eggs, their fecundity was estimated as the number of eggs produced. From the first day after the blood feed, all mosquitoes were checked daily to assess post-feeding survival as described (section 2.7). Survival was monitored until day 10 after blood feeding, at which point all mosquitoes in infected groups were killed and examined for infection. After death, the body size of each mosquito was estimated as described (section 2.7) by measuring wing-length.

6.3.7 Estimating parasite infection in mosquitoes

Mosquitoes fed gametocyte-infected blood were killed and dissected 10 days after feeding to determine if they were infected (the presence of oocysts on the midgut). Mosquito midgut dissection and determination of oocyst infection intensity were carried out as described in Chapter 5 (section 5.3.5).

6.3.8 Quantifying mosquito energetic reserves

Colorimetric-based biochemical analyses were performed to assess the levels of energetic reserves in mosquitoes exposed to different host PCV and malaria infection treatments. Lipid abundance in mosquito bodies was estimated using the vanillin test, and glycogen and glucose abundance estimated from the anthrone test (Van Handel & Day, 1988). The procedure for preparing mosquito samples for biochemical analysis was based on a minor

modification of previous protocols (Van Handel, 1985a; Van Handel, 1985b): the volume of anthrone solution was reduced (Aboagye-Antwi & Tripet, 2010). All mosquitoes in the infectious blood group were killed on day 10 (to detect mature oocysts), but those in the uninfected control group were kept until the natural date of death. The bodies of dead mosquitoes, including the midgut if removed, were stored at -20°C in individual 1.5ml microfuge tubes. For analysis, 0.2ml of sodium sulphate solution (2% concentration) was added to each tube and the mosquito body was homogenised using a non-stick rod (Fisher scientific, Kontes, 749521-0590). Tissue from individual mosquitoes was washed into microfuge tubes with 750 ul chloroform- methanol (1:1) solution. Tubes were then centrifuged (5268 *xg*, 1 min) and the resulting supernatant transferred into clean 15 ml tubes. The pellets formed during centrifugation were retained for glycogen analysis and supernatant was used for lipid and sugar estimations. The supernatant was centrifuged again (5268 *xg*, 1 min) and the top fraction was used for sugar analysis and bottom fraction portion was kept for lipid analysis. (Van Handel, 1985b; Van Handel, 1985a).

Sugar and glycogen concentrations in mosquito tissues samples were estimated from the same standard curve which was obtained from anhydrous glucose solutions made by serial dilution of a stock solution of 1mg/ml (8 different concentrations prepared: 32.2, 16.1, 8.0, 4.0, 2.0, 1.0, 0.5 and 0.25 ug/ml). Linear regression analysis was used to estimate the relationship between the known concentrations of these prepared standard samples and their absorbance at 630 nm, measured in an ELISA plate reader [MRX Revelation TC Absorbance Elisa Plate Reader, USA]. The published protocol recommended a wavelength of 625nm for this test but the ELISA reader which was used in this experiment had only a 630nm filter. The accuracy of this wave length (630nm) was confirmed by checking the original anthrone standard curve (Van Handel & Day, 1988; Van Handel, 1985a). Mosquito samples for sugar analysis were evaporated in a heated block at 90-100 °C until the solvent reach 0.1-0.2 ml level. Three ml of anthrone reagent was added to each tube and mixed. The mixture was re-heated for 17 min at 90-100 °C again. After cooling, absorbance (range of green colour) was measured at 630 nm by an ELISA plate reader (MRX Revelation TC Absorbance Elisa Plate Reader, USA) against a reagent blank. The same process was followed for measurement of glycogen from the pellet , except the heating step.

Lipid concentrations in mosquito tissue samples were estimated from a standard curve which was obtained from soybean oil in chloroform made by serial dilution of a stock solution of 1mg/ml (7 different concentrations prepared: 5, 10, 20, 40, 80, 160, and 320 ug/ml). Linear regression analysis was used to estimate the relationship between the known concentrations of these prepared standard samples and their absorbance at 540 nm, using an ELISA plate reader (MRX Revelation TC Absorbance Elisa Plate Reader, USA). The previous protocol recommended a wavelength of 490nm, for which no filter was available. A wavelength of 540 nm was found to be satisfactory after comparison with a vanillin standard curve (Van Handel & Day, 1988). Briefly, for lipid analysis the solvent was placed in the heating block at (90-100 °C) to evaporate the solvent completely. Then 0.2 ml of sulphuric acid (95- 98%) added to tubes and re-heated them for 10 minutes for converting the unsaturated lipids to water soluble sulphuric acid derivatives (Van Handel, 1985b). After adding vanillin- phosphoric acid reagent and cooling, the reddish colour were read in 540 nm by an ELISA plate reader (MRX Revelation TC Absorbance Elisa Plate Reader, USA) against a reagent blank. Correlation coefficients (R^2) of 0.98 or higher were taken as acceptable for each experimental replicate.

6.3.9 Statistical analysis

Statistical analysis was carried out to assess the influence of host blood PCV, malaria infection status and their interaction on four indices of mosquito fitness: blood meal size, oviposition rate (probability of laying eggs), fecundity (number of eggs laid), and survival up until day 10 (day of dissection for infected mosquitoes). In initial analyses, all mosquitoes fed on gametocyte–infected blood were considered to be in the 'infected blood' treatment. While all mosquitoes in these groups were exposed to gametocytes, not all went on to develop infection as evidenced by oocysts. Thus to distinguish the impacts of exposure to parasites from direct infection, further analyses were conducted on the group fed on infected blood to assess whether fitness varied between mosquitoes that did and did not go on to develop oocysts. This analysis was possible only for mosquito blood meal size, oviposition rate and fecundity. It could not be done for survival as the oocyst infection status of mosquitoes that died prior to dissection day (10 days post feeding) could not be directly assessed.

Oviposition rate and oocyst infection status are binomial variables ('yes' or 'no'). These data were analysed using generalised linear mixed models (glmer) in the R statistical software package (v.2.12.2 (Crawley, 2007)). In these analyses, the main treatment effects of blood PCV and blood infection status (*P. falciparum* gametocyte infected or uninfected) were fitted as fixed explanatory variables. Mosquito wing length was also fitted as an additional explanatory factor. In addition to these fixed effects, experimental replicate was fitted as a random effect. In the first step, a maximal model was tested, including all main and random effects and the interaction of blood infection status and PCV. All non-significant terms were sequentially removed by backwards elimination to yield the minimal, statistically significant model (Crawley M.J., 2010). The significance of terms in these nested models was tested by performing likelihood ratio test (LRT) (Bolker, 2008). A similar approach was adopted to test the significance of these treatment effects to continuous response variables such as blood meal size and fecundity, using the generalised linear mixed effects procedure (lmer) in the R statistical software (v.2.12.2 (Crawley, 2007)).

Survival analysis was conducted using the Cox proportional hazards model in the R statistical software (v.2.12.2 (Crawley, 2007)). In this analysis, a frailty function was used to integrate the random effect of replicate (three experimental replicates) into the Cox model, with blood PCV, blood infection status or infection, their interaction and mosquito wing length fitted as fixed effects. From this maximal model, non-significant terms were sequentially removed by backwards elimination to reach the minimal statistically significant model (Bolker, 2008). As comparison between mosquito survival in infected and control groups was only possible until the day of oocyst detection (when all mosquitoes in infected groups were sacrificed and dissected), this analysis was restricted to estimating variation in mosquito survival up to 10 days after blood feeding. Under field conditions, naturally infected *An. gambiae* s.s. require 10-14 days at a constant temperature of 26^{o}C (extrinsic incubation period of the parasite (Baton & Ranford-Cartwright, 2005b)) before they can transmit *P. falciparum* to a new human host, and the period of time is decreased at higher temperatures. Thus the survival of mosquitoes through this period has critical epidemiological relevance.

Analysis was also conducted to assess how energetic resources varied between mosquitoes in the different blood treatments. As samples from mosquitoes in the

infected and uninfected groups were taken at different points in their life (infected mosquitoes at day 10 post feeding, uninfected mosquitoes on the day of their natural death), it was not possible to directly test how mosquito energetic reserves were influenced by parasitism. However, analysis was conducted to evaluate the impact of blood PCV on energetic reserves within the infected and uninfected groups respectively; with the prediction being that resource depletion in mosquitoes fed on low PCV blood would be greater in mosquitoes also exposed to parasites. For this, separate statistical analysis was conducted on mosquitoes in the infected and control groups, with the main explanatory variable of blood PCV being tested for an association with lipid, glucose and glycogen mass in these mosquito groups respectively. Mosquito body size was fit as additional explanatory variable, with experimental replicate treated as a random effect. These analyses were conducted using the generalised linear mixed effects procedure (lmer) in the R statistical software (v.2.12.2 (Crawley, 2007)).

6.4 Results

Seven replicates of this experiment were conducted. However, successful parasite infection (as evidenced by oocysts on mosquito midguts) was only achieved in three replicates. Here, analysis was restricted only to these 3 replicates in which infection was achieved.

6.4.1 Blood meal size

The impact of parasite infection (presence of gametocytes in the blood meal) on mosquito blood meal size was significantly influenced by host PCV status (PCV × blood infection status = X^2_1= 12.13, P<0.001, N=850, Figure 6.1). Separate analysis of mosquitoes feeding on different blood meal PCVs subsequently revealed that mosquitoes feeding on blood of normal PCV (50%) obtained larger blood meals from uninfected blood than infected blood (X^2_1= 19.28, P<0.001, N=467). However, when blood PCV was low (15%), mosquito blood meal size was similar on infected and uninfected blood (X^2_1= 0.20, P=0.66, N=383). Mosquito blood meal size was significantly and positively related to mosquito body size in experiments with both high and low PCV blood (Normal PCV: X^2_1= 27.34, P<0.001; Low PCV: X^2_1= 29.59, P<0.001).

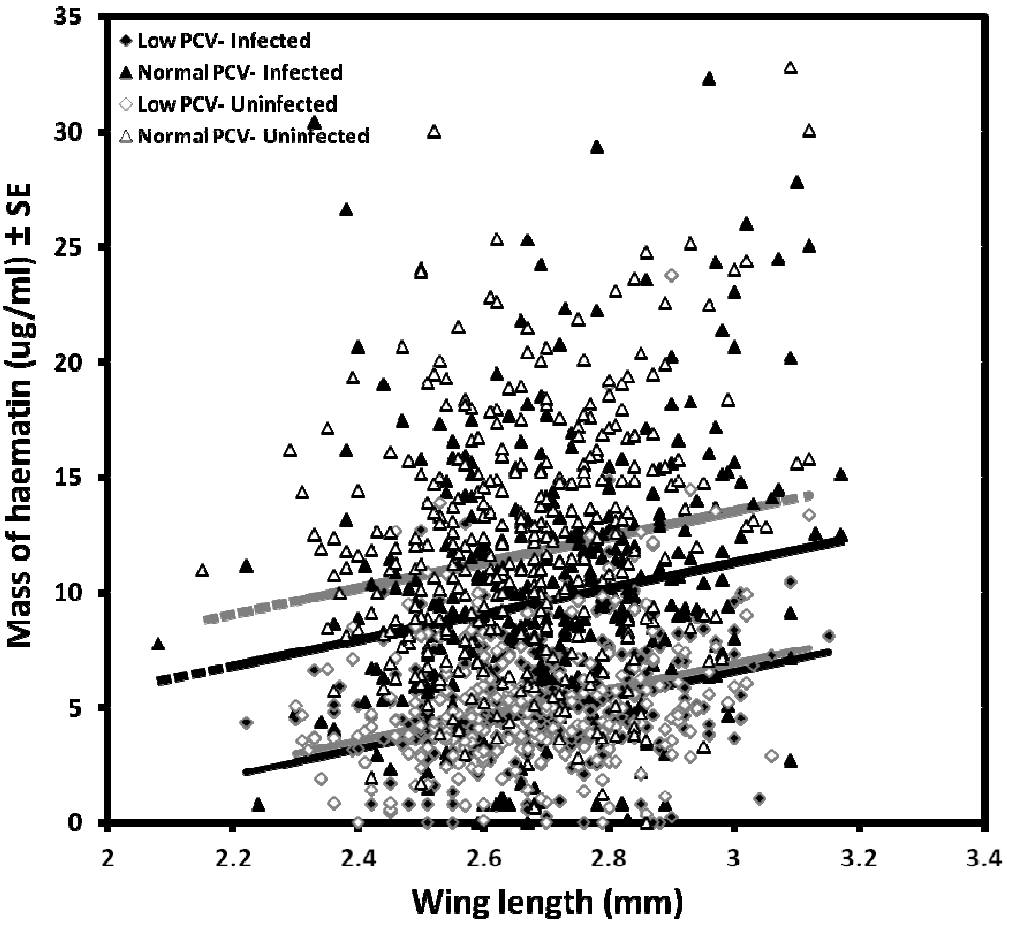

Figure 6.1. Relationship between mosquito body size and the size of blood meal obtained for four different blood types: gametocyte infected, normal PCV (50%) = black triangles (▲), uninfected normal PCV - white triangles (Δ), gametocyte infected low PCV (15%) - black diamonds (♦), and uninfected low PCV (15%) – white diamonds (◊). The solid lines represent the best fitted regression lines for low PCV blood fed mosquitoes and dashed lines represent the best fitted regression lines for normal PCV blood fed mosquitoes. The lines for mosquitoes fed on gametocyte-infected blood are black and for mosquitoes fed on uninfected blood are gray. Low PCV gametocyte infected and uninfected lines overlap and so are not clearly distinguishable from one another (N=850, lmer, R 2.12.2).

Secondary analysis was conducted on mosquitoes fed on infected blood to test if blood meal size was significantly different between those that did and did not develop oocysts subsequently. In this group, the relationship between oocyst presence and blood meal size depended on blood PCV (Oocyst infection × PCV: X^2_1= 4.40, P= 0.03, N= 179, Figure 6.2). Overall, there was no statistical difference in blood meal size between mosquitoes that did and did not develop oocysts after feeding on blood of normal (Normal PCV: X^2_1= 0.48, P=0.49, N=86), or low blood PCV (X^2_1= 0.32, P=0.56, N=93). However, there was a tendency for oocyst-infected mosquitoes to have taken larger blood meals in the normal PCV group, whereas the opposite trend was apparent in the low PCV group (oocyst-infected mosquitoes had taken smaller blood meals), which could have generated the

borderline statistical interaction between oocyst presence and PCV for mosquito blood meal size. Mosquito body size was positively correlated to blood meal size in all mosquitoes exposed to infected blood (Normal PCV: X^2_1= 29.45, P<0.001; Low PCV: X^2_1= 43.41, P<0.001) in this secondary analysis.

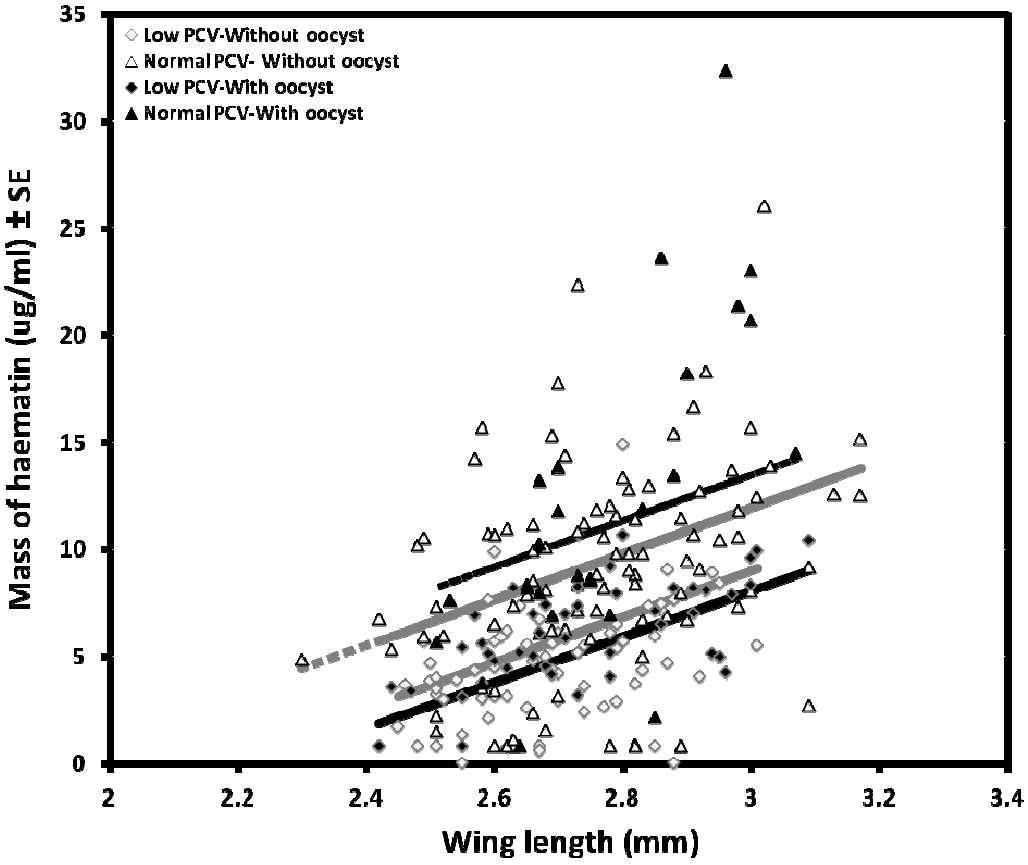

Figure 6.2. Relationship between mosquito body size and size of blood meal (measured as mass of haematin) for four different groups (mosquitoes infected with oocysts, or not infected, fed bloodmeals of low and normal PCV): mosquitoes infected with oocysts, having fed on normal PCV (50%) are shown as black triangles (▲), mosquitoes that did not develop oocysts having fed on normal PCV (50%) as white triangles (Δ), mosquitoes infected with oocysts, having fed on low PCV (15%) blood are shown as black diamonds (♦), and mosquitoes that did not develop oocysts having fed on low PCV (15%) blood as white diamonds (◊). The solid lines represent the best fitted regression lines for mosquitoes fed on low PCV blood and dashed lines represent the best fitted regression lines for mosquitoes fed on normal PCV blood. The lines for mosquitoes that developed oocysts are black and for mosquitoes that did not develop oocysts are gray (N=179, lmer, R 2.12.2).

6.4.2 Oviposition rate

The number of mosquitoes taking a blood meal and the numbers that subsequently laid eggs are shown in Appendix 8.6 , Table 8.10. The raw data (Figure 6.3) show a wide range of oviposition rates. The impact of parasite infection (gametocytes in the blood meal) on

mosquito oviposition was not significantly influenced by blood PCV (Infection x PCV: X^2_1= 0.58, P= 0.44, N=964). However, mosquito oviposition was significantly influenced by the three main effects of blood PCV (greater with low PCV blood, X^2_1= 7.18, P= 0.007), blood infection status (greater with infected blood, X^2_1= 11.26, P<0.001) and mosquito wing length (positively correlated, X^2_1= 46.01, P<0.001) (Table 6.1). In general, mosquitoes that took a blood meal of low PCV had a higher oviposition rate than mosquitoes fed a blood meal at normal PCV. In both groups, mosquitoes feeding on infected blood had a higher oviposition rate than those feeding on non-infected blood. Mosquito oviposition rate was also positively correlated to mosquito body size (Table 6.1, Figure 6.4). The four groups are shown separately (Figure 6.4). In addition, for ease of interpretation the raw data for oviposition is shown in Figure 6.3.

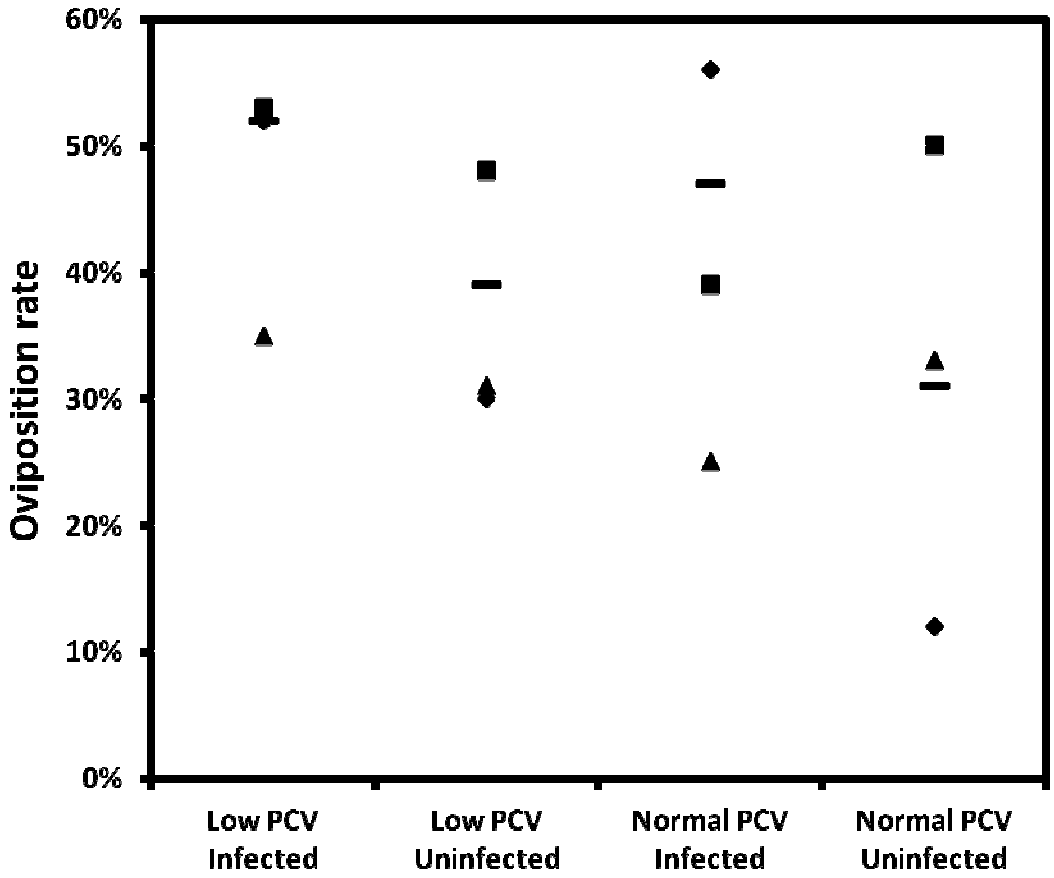

Figure 6.3. Oviposition rate of mosquitoes fed blood of low and normal PCV, containing Gametocytes or no gametocytes. Three replicates of each treatments show as black triangles (▲), black Squares (■) and black diamonds (♦), Median oviposition rate among replicates shows as black thick short lines (-).

Model Parameters	ß estimation	S.E.	*P* value
Intercept	-7.7031	1.16	*P*< 0.001
Wing	2.8007	0.43	*P*< 0.001
Blood uninfected	- 0.4011	0.14	*P*= 0.004
PCV 50%	- 0.4211	0.14	*P*= 0.003

Table 6.1. Logistic regression parameter estimations of the oviposition rate best model with three fixed effects (mosquito wing length, blood infectious status and blood PCV).

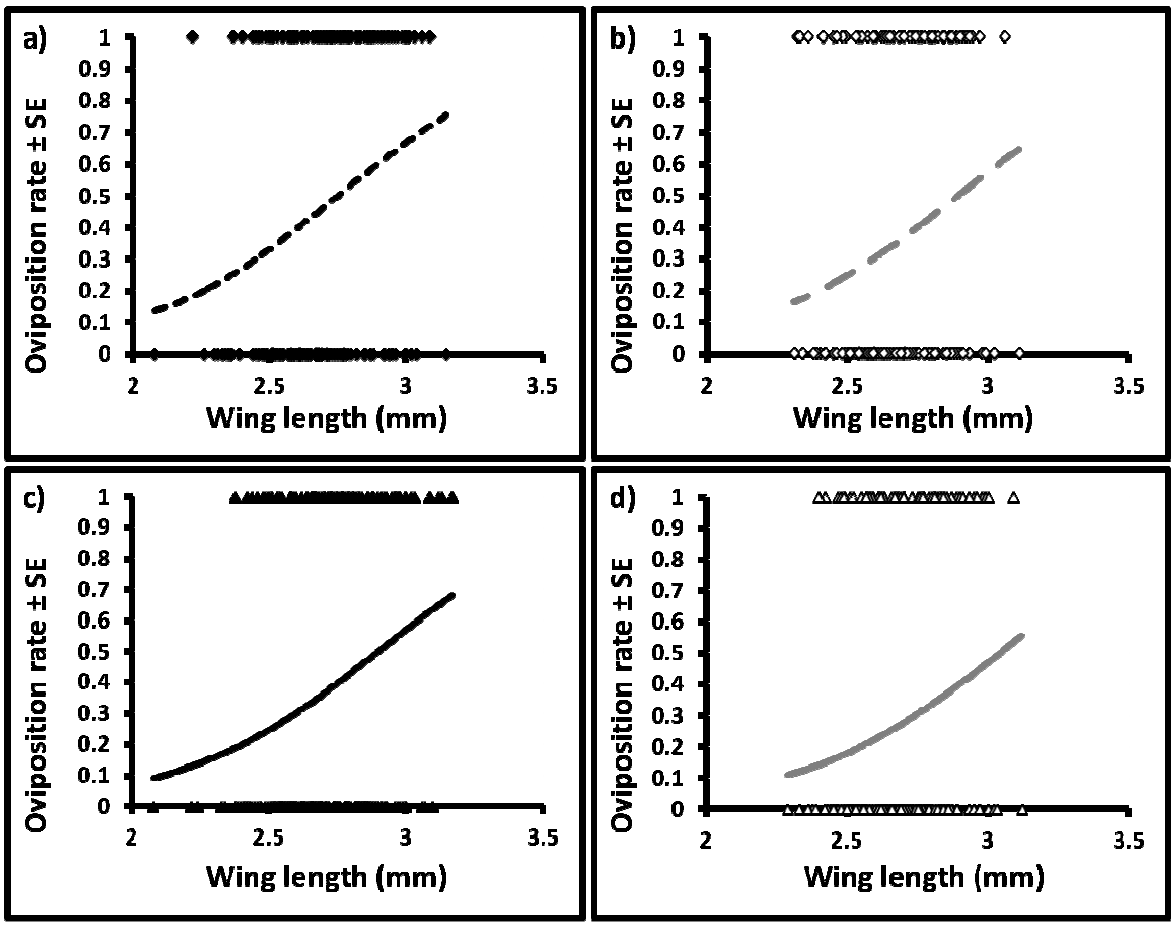

Figure 6.4. The relationship between the size of *An. gambiae* s.s. and oviposition rate after feeding on blood of different types. Points represent mosquitoes that did or did not lay eggs . The logistic regression line shows the best fit statistically association based on estimated parameters of the model (N=964). The four panels represent a) mosquitoes fed on gametocyte-infected blood at low PCV (15%) as black diamonds (♦) with dashed black logistic regression line, b) mosquitoes fed on uninfected blood at low PCV (15%) as white diamonds (◊) and dashed gray logistic regression line, c) mosquitoes fed on gametocyte-infected normal PCV (50%) as black triangles (▲) with solid black logistic regression line and d) mosquitoes fed on uninfected blood at normal PCV (15%) as white triangles (Δ) with solid gray logistic regression line.

Within the group of mosquitoes fed on gametocyte-infected blood, the probability of oviposition was significantly different between mosquitoes that did and did not become

infected with oocysts ten days later (X^2_1= 4.45, P= 0.03, N=179), and was positively correlated with body size in both groups (oocyst infected and uninfected, X^2_1= 15.88, P<0.001, Figure 6.5). Mosquitoes infected with oocysts had a marginally higher oviposition rate than those that had fed on gametocyte-infected blood but that did not subsequently develop oocysts. In this parasite-exposed group of mosquitoes, oviposition rate was not significantly related to blood PCV (X^2_1= 0.02, P= 0.89), or its interaction with oocyst development (PCV × oocyst presence: X^2_1= 2.64, P= 0.10).

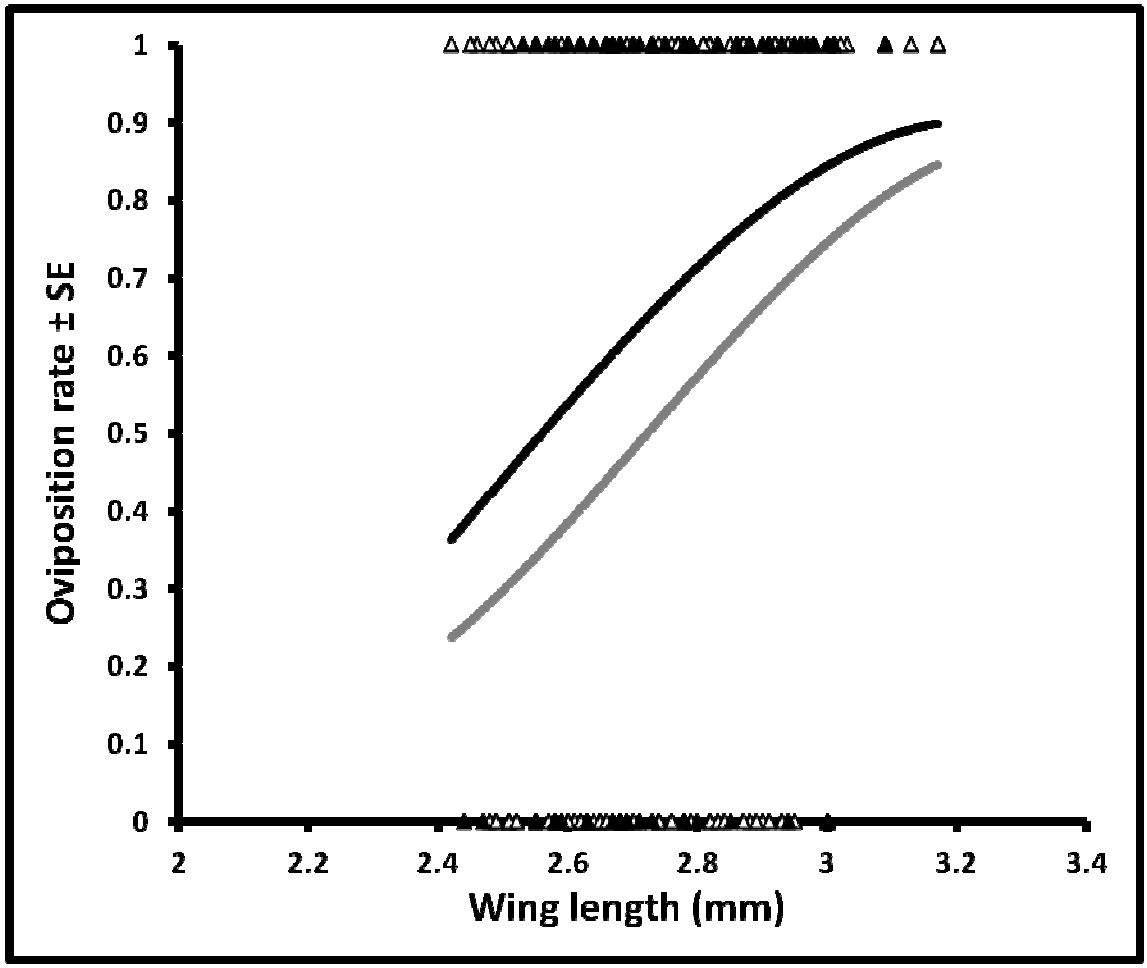

Figure 6.5. The predicted relationship between the size of *An. gambiae* s.s. and oviposition probability after feeding on *P. falciparum* infected blood. Points represent mosquitoes which did or did not lay eggs laid following a blood meal (low and normal PCV pooled, N=179). Oocyst-infected mosquitoes are shown as black triangles (▲) with a solid black logistic regression line and mosquitoes without oocyst infection are presented as white triangles (Δ) with a solid gray logistic regression line.

6.4.3 Mosquito fecundity

The mean number of eggs, SEM and range of eggs laid by mosquitoes in these experiments are shown in Appendix 8.6 and Table 8.11. Restricting analysis to

mosquitoes that laid at least one egg, there was no evidence that fecundity was related to the infectious status (presence of gametocytes) of their blood meal (X^2_1= 0.38, P=0.54, N=325). However, mosquito fecundity was significantly related to host blood PCV (X^2_1= 10.18, P=0.001, N=325) and mosquito body size (X^2_1= 17.41, P<0.001, N=325). Specifically, mosquitoes feeding on blood of low PCV (15%) laid more eggs than those feeding on blood of normal PCV (50%), and the number of eggs laid increased with increasing body size (Figure 6.6).

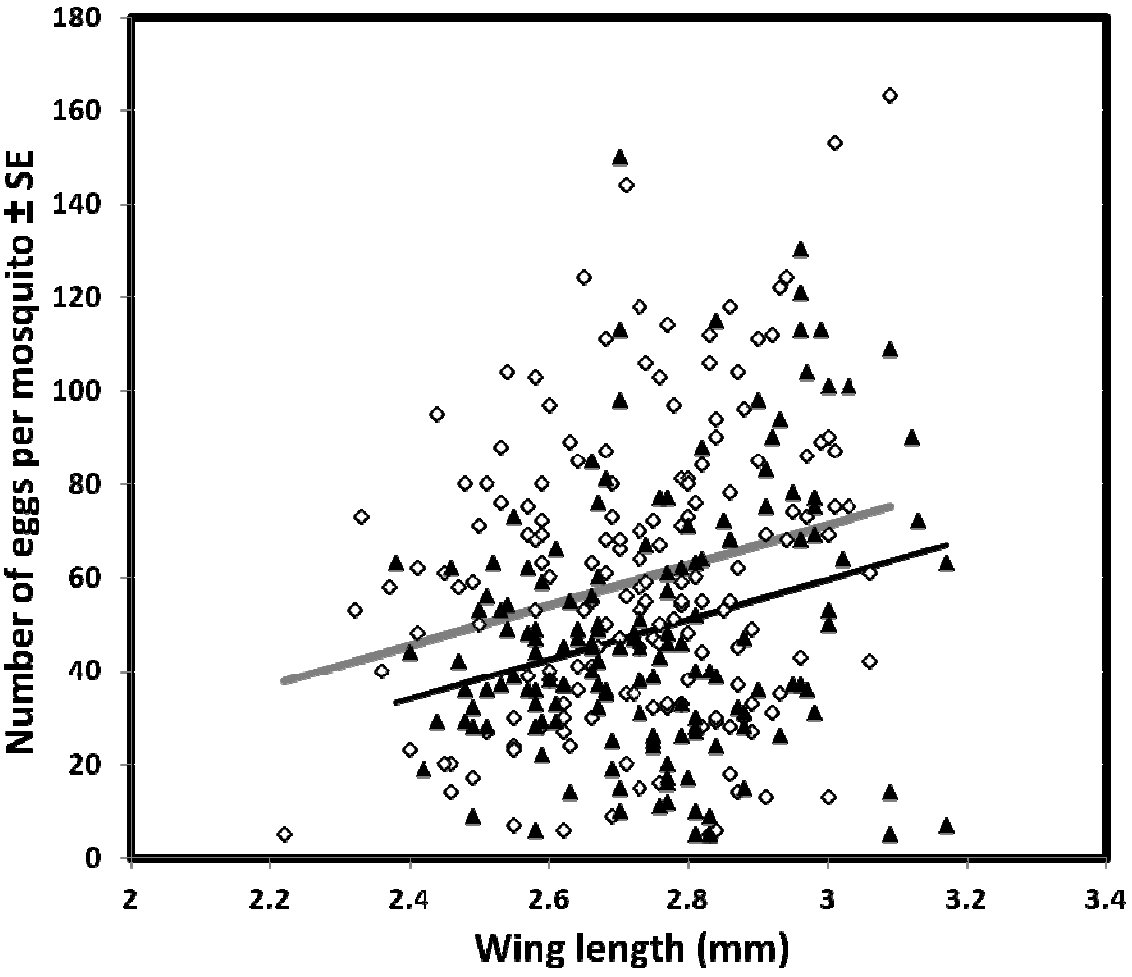

Figure 6.6. Relationship between mosquito fecundity (number of eggs per mosquito) and mosquito body size (wing length) after feeding on blood of two different PCV levels: normal PCV (50%) blood fed mosquitoes are shown as black triangles (▲) and low PCV (15%) blood fed mosquitoes as white diamonds (◊). The black solid line represents the best fitted regression lines for normal PCV blood fed mosquitoes and gray solid line represents the best fitted regression lines for low PCV blood fed mosquitoes (N=325, lmer, R 2.12.2).

Restricting the analysis only to mosquitoes fed on gametocyte-infected blood, there was no evidence that their fecundity was dependent on blood PCV (X^2_1= 1.90, P= 0.16,

N=101), whether they developed oocysts , (X^2_1= 1.54, P= 0.21, N=101) or the interaction of blood PCV and oocyst development (X^2_1= 0.002, P= 0.96, N=101). The only significant predictor of mosquito fecundity in this group was a positive association with body size (X^2_1= 13.33, P<0.001).

6.4.4 Survival

The impact of blood infectious status (presence of gametocytes) on *An. gambiae* s.s. survival varied with blood PCV (PCV * infectious status: X^2_1= 4.05, P= 0.04, N=850). Within the normal PCV group, mosquitoes fed on gametocyte-infected and uninfected blood had similar survival (X^2_1= 0.66, P= 0.42, Odds ratio=0.89, 95% CI= 0.68- 1.17, N=467), and the only variable which had a significant influence on longevity was body size (positive relationship, X^2_1= 5.70, P= 0.02, Odds ratio=0.38, 95% CI= 0.17-0.84, SE= ± 0.40, N=467). However in the group fed on low PCV blood, there was a borderline significant difference in survival between mosquitoes fed on infected and uninfected blood (X^2_1= 3.83, P= 0.050, Odds ratio=1.38, 95% CI= 0.99-1.90, N=383), with those in the infected group having slightly higher survival in the first 10 days following the blood meal (Figure 6.7). There was a marginal relationship between survival and body size in this group (X^2_1= 3.68, P= 0.055, Odds ratio=0.38, 95% CI= 0.14- 1.04, N=383).

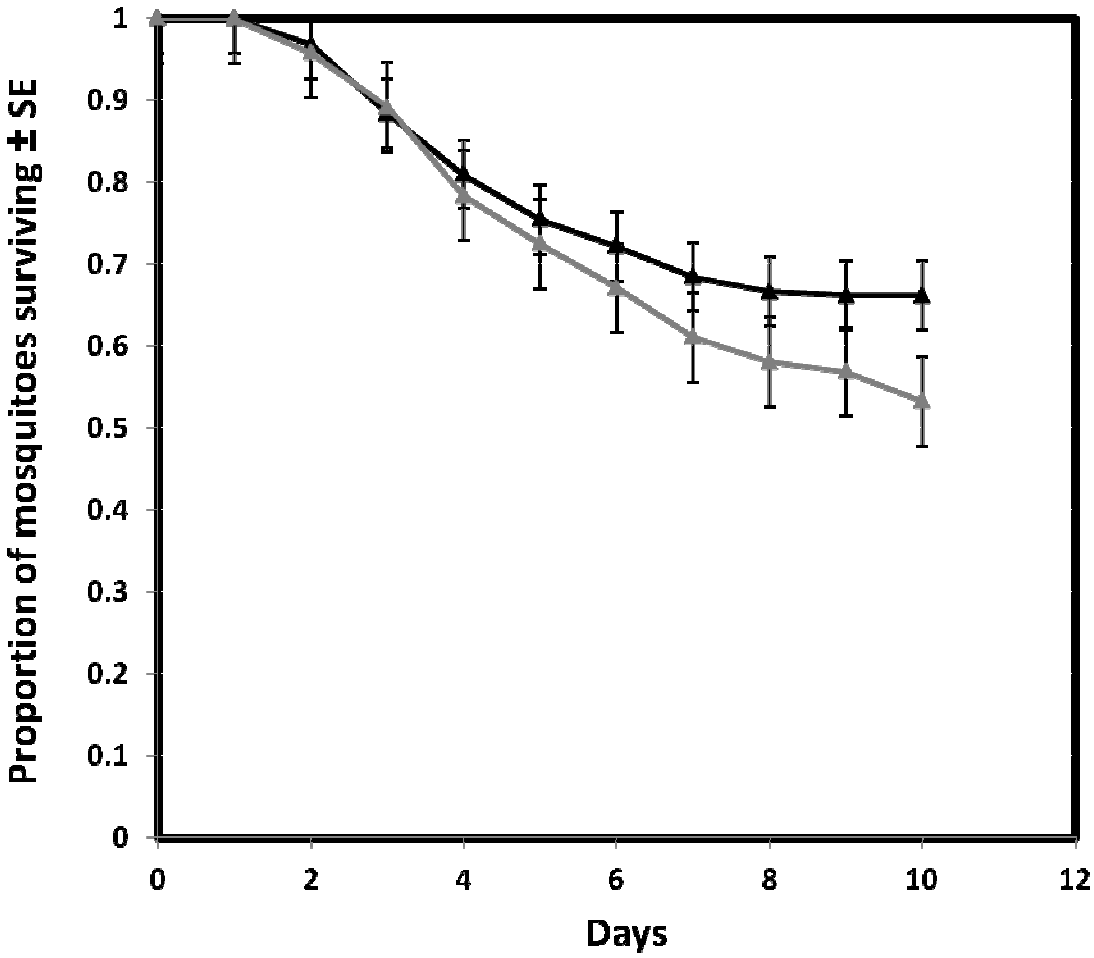

Figure 6.7. Differences in survival of mosquitoes (*An. gambiae* s.s.) during the 10 days after feeding on bloodmeals at low PCV (15%), containing *P. falciparum* gametocytes (solid black line), or non-infected (solid gray line). (P-value= 0.050, Cox's proportional Hazards model, N= 383).

For ease of interpreting the results, the effect of two different PCV levels on survival was also tested in mosquitoes fed a blood meal containing gametocytes and those fed an uninfected blood meal separately. Variation in blood PCV had no impact on the survival of mosquitoes fed either uninfected (X^2_1= 2.43, P= 0.12, Odds ratio=0.79, 95% CI= 0.59-1.06, N=399) or infected blood (X^2_1= 1.43, P= 0.23, Odds ratio=1.20, 95% CI= 0.89- 1.63, N=451). There was no significant association between survival and body size in the uninfected group (X^2_1= 0.24, P= 0.62, Odds ratio=0.80, 95% CI= 0.34- 1.91, N=399), but survival was positively associated with body size in mosquitoes fed infected blood (X^2_1= 12.70, P<0.001, Odds ratio=0.20, 95% CI= 0.08-0.49, SE= ± 0.40, N=451).

In a further analysis, the association between oviposition and survival was investigated by adding mosquito oviposition status as an additional explanatory variable to the survival model. The addition of oviposition did not affect the main conclusions presented above. Specifically, mosquito survival remained significantly influenced by the interaction between blood infectious status and blood PCV (X^2_1= 6.06, P= 0.01, N=850), with mosquitoes fed blood of normal PCV having similar survival on gametocyte-infected and uninfected blood (X^2_1= 0.66, P= 0.42, Odds ratio=0.89, 95% CI= 0.68- 1.17, SE= ± 0.14, N=467), and mosquitoes fed blood of low PCV having marginally higher survival on infected than uninfected blood (X^2_1= 3.83, P= 0.050, Odds ratio=1.38, 95% CI= 0.99-1.90, N=383). However, an additional impact of ovipositon on mosquito survival was detected. The risk of mortality (to day ten post blood meal) in mosquitoes fed on normal PCV blood that laid eggs was approximately 64% lower than in mosquitoes that did not lay eggs (X^2_1= 42.76, P<0.001, Odds ratio= 0.36, 95% CI: 0.26- 0.51, SE= ± 0.16, Cox's proportional Hazards model, N= 467). A similar lower mortality (74%) was also seen in egg-laying mosquitoes fed on blood of low PCV compared to non-laying mosquitoes in this group (X^2_1= 58.98, P<0.001,Odds ratio= 0.26, 95% CI: 0.18- 0.38, SE= ±0.18, N= 383). Thus, mosquitoes that had laid eggs always had better survival than mosquitoes that did not lay eggs, at least up to ten days after the blood meal. This may be due to the positive association between oviposition and body size in these groups (Normal PCV: X^2_1= 26.24, P<0.001; Low PCV: X^2_1= 21.07, P<0.001).

The effect of host blood PCV and oocyst infection rate on mosquito survival could not be directly examined, because all mosquitoes in which oocyst detection was possible had to survive until day 10 post infection (day of dissection), with any mosquitoes dying before this time having undetermined infection status.

6.4.5 Mosquito energetic resources

The energetic reserves of mosquitoes fed on blood meals of different PCV and malaria infection status were examined at different time points. For mosquitoes fed uninfected blood (but with variable PCV), reserve levels were assessed at the time of their natural death. This allowed comparison of the influence of PCV on reserves at death. For mosquitoes fed on gametocyte-infected blood, reserve levels were measured at day 10 after blood feeding, when they were killed for analysis of oocyst infection. As the reserve

levels of mosquitoes fed on infected and uninfected blood were assayed at different time points after feeding, it was not possible to directly compare the impact of 'infection' on these energetic resources. However, it was possible to contrast the impact of blood PCV on energetic levels within the groups of mosquitoes fed on infected, or on uninfected blood.

For mosquitoes fed on uninfected blood, the only significant impact of blood PCV on energetic reserve levels at death was on lipids (X^2_1= 4.89, P= 0.02, N=96). Specifically mosquitoes fed on blood of normal PCV had a higher lipid content at death than those fed on blood with low PCV (Figure 6.8 d). There was no significant difference in the abundance of glucose (X^2_1= 3.68, P= 0.055, N=96) or glycogen (X^2_1= 1.42, P= 0.23, N=96) at death in relation to blood PCV (Figure 6.8 e-f). The abundance of these three reserve types at death was not related to body size (Glucose: X^2_1= 1.90, P= 0.17; Glycogen: X^2_1= 3.00, P= 0.08; Lipid: X^2_1= 0.49, P= 0.48, N= 96, Figure 6.9 d-f).

In contrast to mosquitoes fed on uninfected blood, the energetic reserves of mosquitoes fed on gametocyte infected blood were assayed before their natural death (deliberately sacrificed at day 10 post feeding, to assess oocyst infection rates). At this time point, mosquito lipid content was positively associated with their body size (X^2_1= 6.51, P= 0.01, N=68, Figure 6.9 a) but not with blood PCV (X^2_1= 1.36, P= 0.24, N=68, Figure 6.8 a). In contrast, glycogen levels were marginally higher in mosquitoes fed on gametocyte infected blood of normal than of low PCV (X^2_1= 3.99, P= 0.04, N=68, Figure 6.8 c), but glycogen levels were unrelated to body size (X^2_1= 1.81, P= 0.18, N=68 Figure 6.9 c). The glucose content of mosquitoes that had taken a blood meal containing gametocytes was not significantly related to either blood PCV (X^2_1= 3.43, P= 0.06, N=68, Figure 6.8 b) or to body size (X^2_1= 3.80, P= 0.051, N=68, Figure 6.9 b).

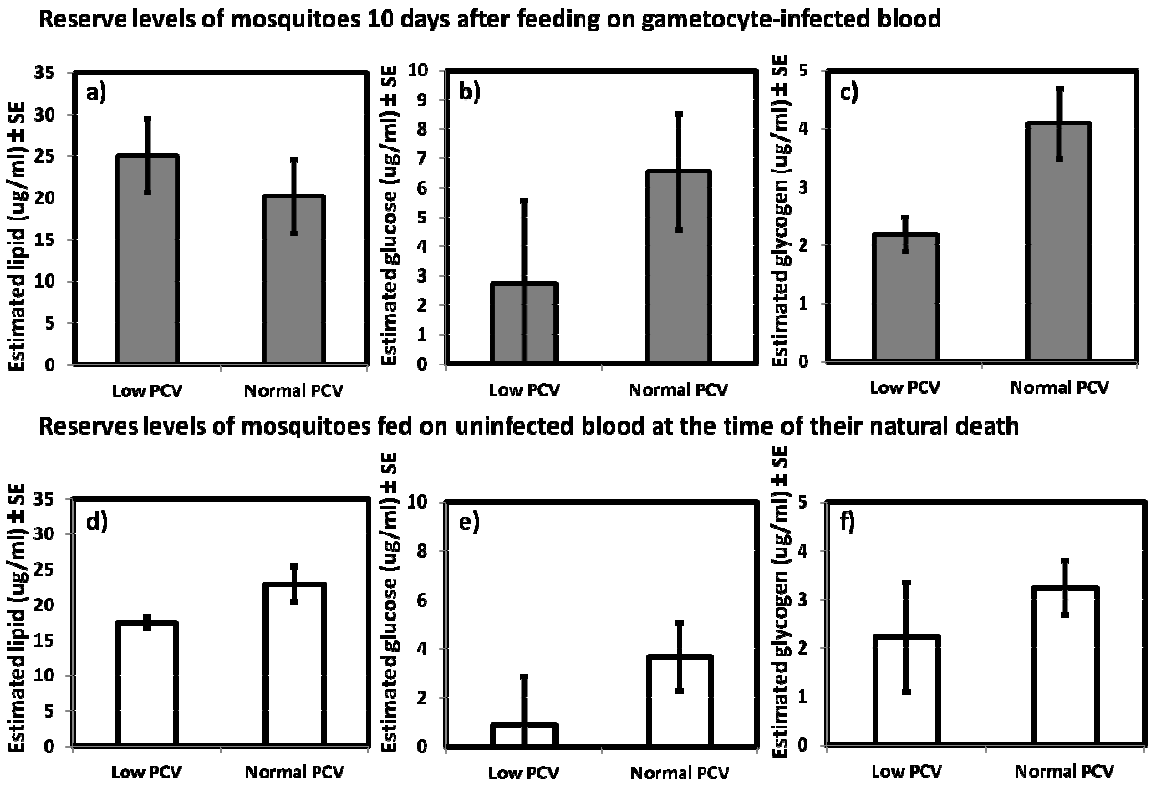

Figure 6.8. Estimation of energetic resources (lipid, glucose and glycogen) in *An. gambiae* s.s. mosquitoes after feeding on blood of different PCV levels (low= 15% and normal PCV= 50%) and malaria-infection status. Reserves in mosquitoes fed on malaria-infected blood were estimated at day 10 post blood feeding, and in uninfected mosquitoes on the day of their natural day of death. Error bars represent ± SE. Panels a-c show the estimation of lipid, glucose and glycogen reserves respectively in mosquitoes fed a gametocyte -infected blood meal (N=68), panels d-f show levels of lipids, glucose and glycogen respectively in mosquitoes fed an uninfected blood meal (N=96).

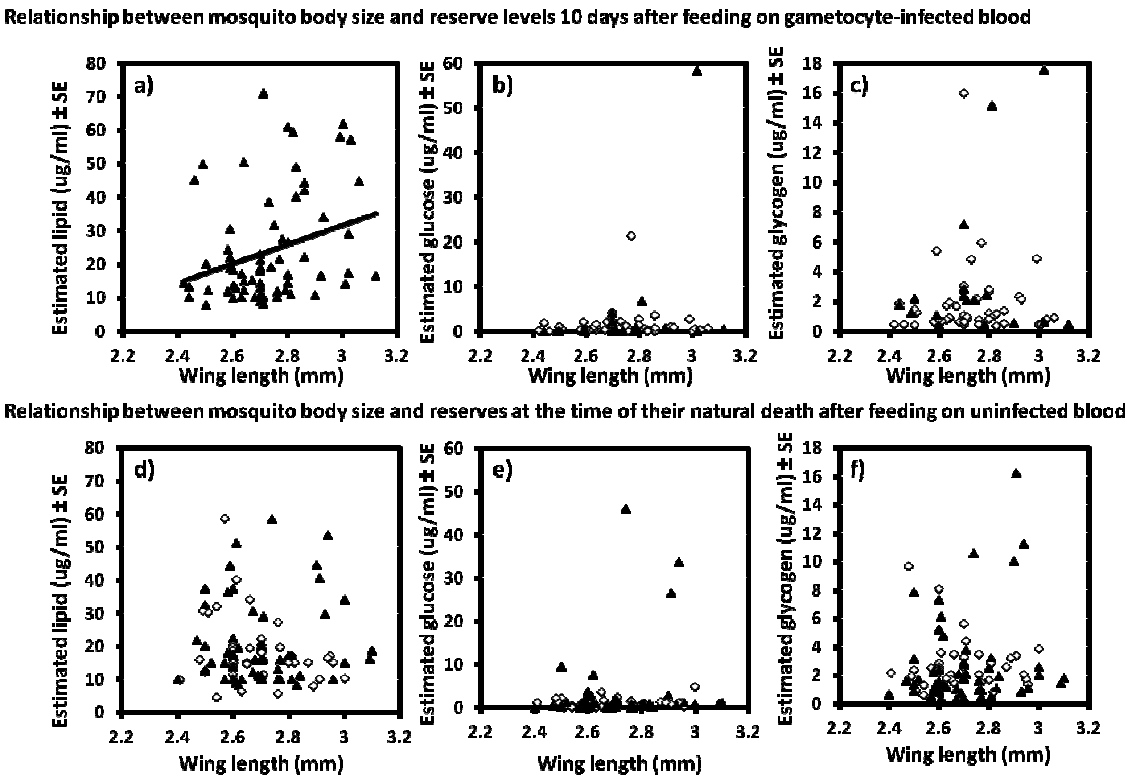

Figure 6.9. The relationship between mosquito body size and their lipid, glucose and glycogen reserves after feeding on blood of different PCV levels (low= 15% and normal PCV= 50%) and malara infection status. Panels a-c show assocations with lipid (black solid line represents the best fitted regression), gluose and glycogen respectively in mosquitoes fed malaria gametocyte-infected blood (assayed at day 10). Panels d-f show assocations between mosquito body size and lipid, glucose and glycogen reserves in mosquitoes fed on uninfected blood of different PCV (assayed at the time of their natural death). Normal PCV (50%) blood fed mosquitoes are shown as black triangles (▲) and low PCV (15%) blood fed mosquitoes as white diamonds (◊).

6.5 Discussion

The primary hypothesis tested in this chapter was whether the virulence of malaria parasites to their mosquito vectors is dependent on the associated host factors that determine the quality of the infectious blood meal - specifically in terms of packed cell volume (PCV). My initial prediction was that any parasite-related virulence as expressed by a reduction in mosquito vector fitness traits such as oviposition, fecundity or survival would be greater in mosquitoes that fed on gametocyte-containing blood of low PCV than those infected from blood with a normal PCV. However, this study found little evidence to support the prediction that the PCV of host blood determines the virulence of malaria parasites to their mosquito vectors. Notably, there was no evidence that either *P. falciparum*-infected blood or the subsequent development of oocysts reduced any measure of mosquito fitness (oviposition, fecundity, survival). While the effects of

exposure to an infection with *P. falciparum* in the blood meal were minimal, host blood PCV and mosquito wing length were repeatedly found to influence most mosquito fitness measures. The overall finding of the current chapter suggested that malaria parasites have little direct effect on mosquito fitness, and thus vertebrate host and mosquito factors may be more important determinants of vector fitness.

Of all mosquito fitness traits examined, the only one that appeared to be negatively affected by exposure to parasites was blood meal size. Significantly smaller blood meals were taken by *An. gambiae* s.s. feeding on gametocyte-infected than uninfected blood of normal PCV, however this effect was not present when infected and uninfected blood was of low PCV. In rodent malaria studies, mosquito blood meal size has also been found to be decreased following feeding on infected hosts compared to non-infected, but interpretation has been complicated by the concomitant reduction in host PCV associated with malaria infection (Ferguson *et al.*, 2003b; Taylor & Hurd, 2001). Specifically it is difficult to disentangle the impacts of infection-related anaemia from exposure to parasites in these studies, whereas there factors could be independently varied here. The reduction of blood meal size associated with infection when PCV was held constant at 50% suggests that the presence of *P. falciparum* gametocytes on their own can alter blood meal size due to unknown mechanisms. However, the similarity of blood meal size in the infected and uninfected group fed on low PCV blood suggests that the presence of parasites in the blood meal does not consistently reduce blood meal size. Thus it seems neither exposure to parasites, or reductions in PCV due to infection, can be conclusively confirmed as the mechanism generating smaller blood meals in mosquitoes feeding on malaria infected hosts.

This study found no evidence that exposure to, or infection by, malaria parasites reduced mosquito reproductive success, in terms of oviposition and fecundity. Oviposition rates were actually higher in mosquitoes fed on infected blood, and amongst this infected group there was (marginal) evidence that mosquitoes that went onto develop oocysts were slightly more likely to lay eggs than those that did not. These observations on oviposition rates agree with previous field research showing that the oviposition rate of *An. gambiae* s,s exposed to natural *P. falciparum* infected blood was approximately six times greater than in mosquitoes fed uninfected blood (Ferguson *et al.*, 2005). Whereas evidence that malaria infection affects mosquito oviposition is limited, there has been

extensive study of its impact on mosquito egg production [fecundity] (Hurd, 2001; Ferguson *et al.*, 2003b; Hogg *et al.*, 1995; Hogg & Hurd, 1995a; Gray & Bradley, 2006; Dao *et al.*, 2010). Several studies of both rodent models (Hacker & Kilama, 1974; Ferguson *et al.*, 2003b; Hurd, 2001; Gray & Bradley, 2006) and natural infections (Hogg *et al.*, 1995) have shown that malaria infection reduced mosquito fecundity. The cause of these observations of mosquito fecundity reduction has been hypothesized to be a direct consequence of parasite infection (Hurd, 2001; Hogg *et al.*, 1995; Gray & Bradley, 2006), and/ or an indirect consequence of a reduction in the PCV of host blood associated with infection (Ferguson *et al.*, 2003b; Taylor & Hurd, 2001). The results of this study support neither hypothesis, with mosquito fecundity being significantly higher on low compared to normal PCV blood, and unrelated to the presence of gametocytes in blood meals or subsequent development of oocysts on mosquito midguts.

It has been suggested that the fecundity reductions due to infection are observed only in unnatural vector–parasite associations used in rodent malaria studies (Ferguson *et al.*, 2003b; Hogg & Hurd, 1995a; Gray & Bradley, 2006; Taylor *et al.*, 1998). While observations made here support this hypothesis in finding no evidence of infection-related fecundity reduction this natural vector-parasite combination, another study of *An. gambiae s.s.* infected with *P. falciparum* under field conditions in Tanzania also reported their fecundity was decreased by infection (Hogg & Hurd, 1997). Thus it cannot yet be concluded that the lack of parasite virulence reported here is wholly due to the parasite-vector combination used. A further possibility is that the mosquito fecundity reductions observed in other studies are actually a product of other characteristics of naturally infected blood, such as the asexual stages of the parasite and/or other plasma components that are not captured in the *in vitro* infection system used here. For example, while mosquitoes are thought to only use nutrients from red blood cells (Briegel & Rezzonico, 1985a; Takken *et al.*, 1998; Nayar & Sauerman, 1975; Dadd, 1980), it is possible that their fecundity is also partially influenced by the composition of amino acids, lipids and proteins in blood plasma (Uchida *et al.*, 1990; Uchida *et al.*, 1998; Uchida *et al.*, 2001; Uchida *et al.*, 2003; Briegel *et al.*, 2002; Atella *et al.*, 2006). In these experiments, all mosquitoes were infected with blood mixtures created from a standard pool of human serum from malaria-naïve individuals. Thus if plasma-related factors are responsible for malaria infection-related decreases in mosquito fecundity, I would not expect to see any

effect in these experiments. Further experiments are required to test the potential role of blood plasma factors on mosquito fitness to test this hypothesis.

There was no evidence that exposure to *P. falciparum* in blood meals reduced *An. gambiae* s.s. survival. In fact contrary to initial predictions, there was instead evidence that mosquitoes fed on infected blood had a marginal survival advantage ($p = 0.050$, low PCV blood treatment only). Given the borderline significance of this effect, it would be premature (given on average, only 43.6% of mosquitoes became infected) to conclude whether this represents a genuine survival benefit of infection without further testing. However, the finding of no reduction in mosquito survival in association with malaria exposure is consistent with those of several other studies of natural mosquito vector-parasite associations (Lines *et al.*, 1991; Robert *et al.*, 1990; Chege & Beier, 1990; Hogg & Hurd, 1997; Boyd, 1940; Anderson & Roitberg, 1999). Whereas numerous laboratory studies using artificial vector-parasite associations have reported that infection reduces mosquito survival (Klein *et al.*, 1982; Gad *et al.*, 1979; Hogg & Hurd, 1995b; Ferguson *et al.*, 2003a; Dawes *et al.*, 2009), this effect is largely absent in studies of natural combinations (Ferguson & Read, 2002b). Given that any reduction in mosquito survival would reduce parasite transmission success (Macdonald, 1957), it has been hypothesized that this type of virulence would only occur in systems that had not co-evolved together, where natural selection would be expected to have minimized any impact of *Plasmodium* infection on mosquito survival.

Although this study provided no evidence of *P. falciparum* virulence in its vectors, or that low blood PCV enhanced virulence, the predicted impact of *P. falciparum* exposure and infection was influenced by host blood in other ways. Notably, mosquito survival in the 10 days following the blood meal was significantly influenced by the interaction between blood infection status and PCV. Mosquitoes fed on blood of normal PCV had similar survival regardless of whether their blood meal was infected or not, but in the low PCV group those fed on infected blood had a slight survival advantage. As the malaria infection status of mosquitoes that died naturally (before dissection on day 10) was not assessed, it is not clear whether this effect was due simply to exposure to parasites in the blood meal and/or to a direct effect of infection (infection rate in low PCV groups= 31%-86%), and the mechanism behind this apparent increase in mosquito survival associated with parasites in a low PCV blood meal is unclear. If parasites are indeed capable of

altering vector survival, as has been suggested in some theoretical models, then an increased likelihood of survival, at least during the sporogonic cycle, would be favoured (Schwartz & Koella, 2001; Frank, 1996; Stone *et al.*, 2011). However, there is currently little evidence to support a significant positive impact of *Plasmodium* on vector survival; the results presented here are the first account of an apparent "increase" in mosquito survival rate during the malaria parasite extrinsic incubation period.

An unanticipated impact of blood PCV in these experiments was its opposing effects on mosquito blood feeding and reproduction as described in other chapters (4). Mosquito oviposition rate and fecundity are known to be positively related to the size of the mosquito blood meal (Briegel & Rezzonico, 1985a; Taylor & Hurd, 2001; Ferguson *et al.*, 2003b; Briegel & Horler, 1993; Hansen *et al.*, 2004; Kokoza *et al.*, 2001). However, the observed impacts of *P. falciparum* infection and blood meal PCV on mosquito blood meal size differed from those on mosquito reproduction. Specifically, larger blood meals were obtained from uninfected blood of normal PCV, followed by infected blood of normal PCV, and then low PCV blood (with no difference between infected and uninfected blood). However, the opposite ranking was observed for oviposition rate, where mosquitoes fed on gametocyte-infected blood of low PCV (which resulted in the smallest blood meals) had the highest probability of laying eggs. This result was also evident for mosquito fecundity: mosquitoes that oviposited after feeding on low PCV blood laid substantially more eggs than those feeding on blood of normal PCV. The presence of gametocytes in the blood meal had no significant impact on fecundity, but in mosquitoes taking a blood meal containing gametocytes, oviposition rates were higher in mosquitoes that went on to develop oocysts than those who did not. The causes of this breakdown in the relationship between blood meal size and fecundity across PCV treatments may be due to an energetic reallocation by mosquitoes and/or the presence of additional serum factors that contribute to mosquito reproduction which are not considered here.

Given that malaria infection had minor impact on *An. gambiae* s.s. fitness (blood meal size) in these experiments, it might also be expected to have had little effect on mosquito energetic reserves. The data were used to test the general prediction that if malaria parasites do limit mosquito resources, the effect of a low quality (low PCV) blood meal on mosquito energetic resources should be greater in mosquitoes exposed to parasites than those who were not. Direct quantitative evaluation of this hypothesis was not possible

because the energetic reserves of mosquitoes fed on infected and uninfected blood were assayed at different points in their life history (e.g. uninfected = at time of natural death, infected = 10 days after feeding), and different parts of the mosquito were available (whole carcases for uninfected mosquitoes, but carcases excluding midguts for mosquitoes fed a gametocyte-containing blood meal). Some preliminary qualitative assessment can nevertheless be drawn from the trends in each group. Here, the impact of blood PCV on the energetic reserves of both infected and uninfected mosquitoes was found to be relatively minor. In uninfected mosquitoes, only the lipid abundance at death was higher in mosquitoes fed on normal rather than low PCV blood; glycogen and glucose levels were similar. As lipid levels are the primary determinant of long term survival, it might be expected that these mosquitoes would live longer which was indeed the case (mosquito long-term survival higher on normal PCV blood). The similarity of glycogen and glucose levels in mosquitoes fed on low and normal PCV blood is perhaps not surprising as these were measured at the time of natural death, when all of these resources may be similarly exhausted as result of general activity, or would have been replenished by *ad libitum* glucose feeding as available to all mosquitoes. In the group fed on gametocyte-containing blood, the only difference in reserve levels at day 10 post feeding was a marginal reduction (P= 0.04) in glycogen abundance in mosquitoes given a low PCV blood meal. The reasons for the observed difference are unclear and may not be biologically meaningful.

Plasmodium infection has been shown to reduce mosquito energetic reserves (e.g. glucose) in *P. cynomolgii* infections in *An. stephensi* (Schiefer *et al.*, 1977). Previous investigations in rodent malaria model systems found no evidence that *Plasmodium* infection reduced mosquito energetic reserves, and infection was associated with an increased abundance of sugars (Rivero & Ferguson, 2003). In the rodent malaria work, mosquito energetic reserves were measured in infected and uninfected mosquitoes at the same time point post blood feed (day 7 and day 14 post infectious feed), allowing comparisons to be made. It was not possible to compare in a similar way reserve levels in mosquitoes fed on blood containing *P. falciparum* gametocytes, and mosquitoes fed on uninfected human blood in current experiment, but the fact that differences in mosquito energetic reserves due to PCV variation were not more pronounced in these two groups of mosquitoes suggests that resources are not very limited in either of these groups. This hypothesis is consistent with my general observation that *P. falciparum* is not virulent in

*An. gambiae s.*s., but further investigation is required to assay repeatedly the energetic levels of infected and uninfected mosquitoes at the same time points through the full course of infection to confirm this.

While the influence of blood PCV on mosquito fitness traits and energetic reserves was variable, and the impacts of *P. falciparum* exposure and infection were largely absent, the most consistent predictor of *An. gambiae* s.s. fitness was mosquito body size. In accordance with most previous studies, mosquito body size was positively correlated with blood meal size (Hurd *et al.*, 1995; Takken *et al.*, 1998; Briegel, 1990a; Hurd *et al.*, 1995; Hogg & Hurd, 1995a), oviposition rate (Lyimo & Takken, 1993; Briegel, 1990a), energetic reserves (Nasci, 1986; Rivero & Ferguson, 2003), fecundity (Andersson, 1992; Lyimo & Takken, 1993; Briegel, 1990b; Yaro *et al.*, 2012) and survival (Ameneshewa & Service, 1996; Ferguson & Read, 2002a). These overwhelming impacts of mosquito body size suggest that it may be intrinsic properties of mosquito vectors themselves, rather than host haematological or parasite factors, that have the greatest impact on their ultimate fitness. However, the same conclusion may not be made for mosquito vectorial capacity. While mosquito fitness is determined both by their fecundity and survival, the transmission success of malaria parasites is only dependent on mosquito survival (Dawes *et al.*, 2009; Macdonald, 1956a; Macdonald, 1956b; Aboagye-Antwi *et al.*, 2010; Stone *et al.*, 2011). In these experiments, both mosquito body size and blood PCV had significant impacts on mosquito survival found to influence mosquito longevity (results presented here and Chapter 4). While PCV was not found to impact the survival of mosquitoes through the parasite's extrinsic incubation period (1st 14 days after feeding, Chapter 4), it did significantly influence long term survival beyond this minimal threshold for survival which could influence transmission by affecting the number of biting opportunities for infected mosquitoes.

While this study found no evidence for the virulence of *P. falciparum* in its *An. gambiae* s.s. vector, only a few possible manifestations of virulence were examined. There are numerous other ways by which malaria parasites could cause harm to their mosquito vectors that may not be evident under these highly controlled settings. For example, possible deleterious effects of parasites on vectors include impairment of biochemical, molecular and physiological processes (Ramasamy *et al.*, 1997; Seitz *et al.*, 1987; Vaughan & Turell, 1996; Baton & Ranford-Cartwright, 2007; Jahan *et al.*, 1999; Beier, 1998; Hurd *et*

al., 1995) which could amongst other things increase their susceptibility to other infections (Seitz *et al.*, 1987; Vaughan & Turell, 1996), or their ability to avoid predation which has not yet investigated or host defensive behaviour (Rossignol *et al.*, 1984; Wekesa *et al.*, 1992; Anderson *et al.*, 1999b; Koella *et al.*, 1998). None of these potential costs of parasitism would be evident under the ideal, standardized insectary conditions in which these experiments took place. Furthermore, mosquito fitness was assayed after taking only one blood meal, whereas in nature *An. gambiae* s.s. will blood feed repeatedly every 2-4 days until death (Romoser *et al.*, 1989; Briegel & Horler, 1993; Ramasamy *et al.*, 2000). If the cost of parasitism is cumulative, and only evident after numerous blood meals and/or increases with age, it would not be detected in these experiments. Finally, the failure to detect virulence in this system could have been influenced by the artificial nature of the *in vitro* infection system. Mosquitoes were infected not from a live host, whose blood composition and behaviour may be influenced by malaria in many complex ways (Ferguson *et al.*, 2003b; Wekesa *et al.*, 1992; Hogg & Hurd, 1997; Okech *et al.*, 2004a; Riehle *et al.*, 2006), but from an artificial membrane feeding device with cultured gametocytes mixed with human blood that had been stored prior to use. A strength of this system provides an ideal method for infecting mosquito vectors with human pathogens without involving human subjects, and permits standardization of crucial determinants of infectivity like parasite density and immune factors in serum (Mitri *et al.*, 2009; Chadee & Beier, 1997; Aurelie *et al.*, 2007; Cohuet *et al.*, 2006; Dong *et al.*, 2006; Baton *et al.*, 2006). However, if the virulence of parasites to mosquito vectors is in part due to associated serum or host factors and not the parasites themselves, it would not be picked up in this experimental design. In the future, a new study system with blood taken from naturally anaemic patients, or from malaria-infected patients and/or multiple feeding designs would be necessary to confirm whether the laboratory based findings would also be true under more natural conditions.

6.6 Conclusions

This study tested the hypothesis that the effect of *P. falciparum* on the fitness and transmission ability of their mosquito vectors is dependent on host haematological factors such as PCV. The initial hypothesis was that any virulence displayed by *P. falciparum* in its *An. gambiae* s.s. vector, as expressed by a reduction in mosquito fitness relative to uninfected controls, would be more pronounced under conditions where the

red cell density (measured by PCV) of the infecting blood meal was low rather than normal. Overall *P. falciparum* was found to have no negative impact on any proxy of mosquito fitness (oviposition, fecundity and survival). Furthermore rather than increasing the cost of parasitism, there was some evidence that low PCV blood provided a fitness advantage to infected mosquitoes, as *An. gambiae* s.s. laid a higher number of eggs after feeding on low rather than normal PCV blood, both when those blood meals contained infectious gametocytes and when they were not infected.

One possible hypothesiss arising from these study findings is that the apparent 'advantage' of low PCV is just the result of a life-history adaption in mosquitoes, who increase their relative investment in early reproduction (egg production after 1st blood meal) if the blood meal is of poor quality for long-term survival (Stearns & Stearns, 1992). If this was the case, it might be impossible to observe a 'cost' after one blood meal. Real associations would require measurement of total lifetime egg production with repeated blood feeds on the same PCV blood throughout mosquito's life. There is some life history theory [e.g. Daphnia studies, (Chadwick & Little, 2005; Stearn & Koella, 2008)] that indicates that infected invertebrate hosts should increase their investment in early reproduction if their long-term survival is compromised by parasitism. A reduction in survival following feeding on blood of low PCV blood would support this theory.

Chapter 7. General Discussion

In this chapter, a summary of the major conclusions from this thesis and their support for my initial research hypotheses about the role of anaemia in malaria transmission are presented. These findings have implications for several areas including malaria epidemiology and evolution which will be discussed.

7.1 Principal findings

Four key hypotheses regarding the potential for human red cell density (PCV) to influence malaria transmission were experimentally tested in this thesis. All of these experiments required conducting experimental infections of *An. gambiae* s.s. mosquitoes with *P. falciparum* parasites grown in vitro using a membrane feeding system. Prior to experimentally testing the main research hypotheses (Chapters 4-6), pilot work was conducted to evaluate the role of laboratory conditions in generating variability in some of main biological outcomes (mosquito fitness) under study. Specifically, the dependence of mosquito fitness measurements on the host individual from which blood was taken (standardized for PCV), and the duration of blood storage prior to experimentation, was examined. This pilot work informed further experiments by showing that 'fresh' blood (obtained 2-3 days after donor bleed, and stored for no more than 7 days before usage in experiments) was optimal for mosquito feeding and fitness, and thus all experiments were conducted with blood of this storage age. Additionally, using blood from a range of different donors introduced relatively small but statistically significant variation in some measures of mosquito fitness. Thus to control for even this minor source of experimental variation, in the rest of my experiments each replicate contrasting blood of different PCV was conducted using a blood sample from the same donor.

7.1.1 Does blood PCV influence mosquito fitness?

As originally hypothesised, reductions in blood PCV commensurate with severe anaemia were associated with a significant reduction in blood meal size. However although blood meal size was reduced, mosquitoes that fed on low PCV blood (15%) exhibited no detectable fecundity cost: they had similar oviposition rates and produced more eggs than those fed on blood of normal PCV (50%). This finding was unexpected, and contrary to my initial hypothesis that mosquito reproduction would be reduced at low blood PCV.

It also yields the surprising finding that blood meal size and fecundity are not consistently related. Possible reasons for this are discussed later on in this chapter (section 7.2.1). Although the causes of this phenomenon are not yet known, it could be interpreted as evidence that host anaemia may benefit mosquito fitness. However, this would only be true if the eggs laid by mosquitoes fed on this resource are of the same quality as those produced from normal blood. Previous studies on insects have shown food-deprived females had offspring of lower quality compared to normally fed females (Jones & Widemo, 2005; Kyneb & Toft, 2006; Frost *et al.*, 2010; Rivero & Casas, 1999; Wheeler & Buck, 1996). To date, there is no information on the effect of host haematological variation on *Anopheles* mosquito egg quality. Thus I hypothesize that eggs generated from low PCV blood may have been provisioned with fewer maternal reserves and may have a lower hatch rate compared to those produced from normal PCV hosts. However, more detailed mechanistic investigations would be required to confirm if the observed effect is generated by an energetic trade-off between fecundity and survival.

7.1.2 Does blood PCV influence parasite infectiousness to mosquito vectors?

Here it was shown that when gametocytes are present in blood at the same density (gametocyte/ml of blood), those in blood of low PCV were more likely to infect mosquitoes than those in blood of normal PCV. In previous studies with naturally-infected hosts there was also some evidence that anaemic hosts were most likely to infect mosquitoes (Drakeley *et al.*, 1999; Nacher *et al.*, 2002). However in natural infections host PCV and gametocyte density are usually highly correlated (Paul *et al.*, 2004; Drakeley *et al.*, 1999; Price *et al.*, 1999; Nacher *et al.*, 2002), so it has been difficult to disentangle which factor is most important for mosquito infection rate. The results obtained here suggest low PCV blood may generate an additional transmission advantage beyond just what can be explained by its association with higher gametocyte densities. A possible mechanism for this would be that male gametes are better able to move through a lower viscosity blood meal (in blood meals of low PCV) to find and fertilise a female gamete.

This suggests that parasite transmission success may be enhanced by the co-occurrence of anaemia in human hosts, either as direct result of malaria infection or other health problems. As anaemia in common within the African populations where *P. falciparum* is transmitted (Green *et al.*, 2011; Trampuz *et al.*, 2003), if effects described here occur in nature this host factor could play a significant factor in enhancing malaria transmission within communities.

In addition to the effects described here, it has been hypothesized that symptoms such as anaemia could provide an additional transmission advantage to malaria parasites by influencing the attractiveness of hosts to mosquitoes; for example, human host cues such as $C0_2$ and lactic acid in the blood or tissues are known to be attractive to mosquitoes (Murphy *et al.*, 2001; Takken, 1991). Thus if anaemia causes hosts to produce more of these compounds (Singhal & Saxena, 1987; English *et al.*, 1997; Marsh *et al.*, 1995; Blumgart & Altschule, 1948), they may be more likely to attract and thus infect mosquitoes.

Further field investigation, comparing the infectiousness of hosts with varying levels of PCV and gametocyte density would be needed to quantify the full impacts of anaemia on malaria parasite infectiousness. However, the experimental results support the crucial prediction from virulence-transmission trade-off theory that some level of parasite virulence in the vertebrate host (anaemia) can enhance malaria transmission success (Levin *et al.*, 1982; May & Anderson, 1983; Ewald, 1983; Stearn & Koella, 2008; Schwartz & Koella, 2001; Day, 2001; Cotter *et al.*, 2011).

7.1.3 Does host PCV influence parasite sporogonic success in mosquito vectors?

Although blood PCV was found to have a significant impact on the infectiousness of parasites to mosquitoes (establishment of oocysts), it had no longer-term impact on the growth of transmission stage sporozoites within these oocysts. Specifically, mosquitoes infected from either low or normal PCV went on to develop a similar number of sporozoites (at least on the mosquito midgut). This suggests that energetic resources in the infectious blood meal are not important for the longer term sporogonic development of parasites. However, these results were reported only after one infectious blood meal.

It is possible that if mosquitoes were repeatedly fed throughout the sporogonic cycle, parasite development and sporozoite abundance would be enhanced in those fed on blood of normal PCV. In accordance with this hypothesis, it was observed that the number of sporozoites developing in an individual *P. falciparum* oocyst was significantly increased when *An. gambiae* s.s. was fed 4 additional (non-infected) blood meals after infection (Dr. L. Ranford-Cartwright, manuscript in preparation). This effect was not apparent when up to 3 additional blood meals were provided, suggesting that although additional blood resources may influence sporozoite production they do so only if provided at high frequency. Additional experiments comparing the sporozoite load in mosquitoes repeatedly feed on blood of varying PCV would be required to predict the full impacts of this haematological variable on malaria parasite sporogonic success.

The current results indicate a single blood meal with different PCV does not have an impact on the total number of sporozoites per midgut, but that does not necessarily correlate with the sporozoite load that ends up in the salivary glands (Ponnudurai *et al.*, 1990). Previous study with naturally infected mosquitoes and experimentally infected mosquitoes (Rosenberg & Rungsiwongse, 1991; Ponnudurai *et al.*, 1990; Gamage-Mendis *et al.*, 1993) found that a proportion (10-15%) of oocyst-infected mosquitoes did not develop salivary gland infections (Gamage-Mendis *et al.*, 1993). The impact of PCV on this proportion of oocyst-infected mosquitoes that become infectious is unknown, and so it is currently not possible to conclude that PCV has no impact on sporogony. In addition, the sporozoites that developed from both PCV treatments are not known to be equally infectious to a host. Further experiments including measurement salivary gland infection following feeding on infectious blood meals at different PCV would allow the influences of host PCV on malaria parasite sporogonic success to be further investigated.

7.1.4 Is parasite virulence to vectors mediated by host PCV?

Plasmodium falciparum infection was generally found to have no harmful impact on any mosquito fitness traits (oviposition, fecundity and survival). The only evidence of a potentially costly effect of parasitism was that mosquitoes obtained smaller blood meals from infected rather than uninfected blood at normal PCV. However as this difference in blood meal size did not translate into a difference in the subsequent mosquito reproduction or survival, it cannot be interpreted as virulence.

There are other ways that PCV might influence parasite virulence to mosquitoes would not be detected by the methodology used here. For example, mosquitoes fed on infectious blood meals of low PCV had lower energetic reserves of glycogen 10 days post blood feed than normal PCV infectious blood. It is possible that, under more 'energetically demanding' conditions such as those found in nature, where mosquitoes have to find oviposition sites, feeding on blood meals of low PCV, that often accompanies blood meals containing parasites, would make these mosquitoes more likely to die. If such activities are more costly when mosquitoes are in natural environment, the influence of host blood PCV could have a substantially larger effect on parasite virulence in the wild compared to under laboratory conditions.

7.2 Questions arising

7.2.1 Why were there contradictory effects of PCV on blood meal size and fecundity?

Numerous studies have demonstrated that mosquito fecundity is positively correlated with blood meal size (Hurd *et al.*, 1995; Hogg *et al.*, 1995; Hogg & Hurd, 1995a; Ahmed *et al.*, 2001). In this study, host blood of low PCV was associated with smaller mosquito blood meal sizes, consistent with previous laboratory studies (Briegel & Rezzonico, 1985b; Ferguson *et al.*, 2003b; Taylor & Hurd, 2001). However contrary to expectation, the smaller blood meals obtained from low PCV blood were associated with higher rather than lower mosquito fecundity. This effect was found for mosquitoes feeding on both malaria parasite-infected and uninfected blood.

A possible explanation for these results as presented previously (Chapter 4) could be that mosquitoes fed on blood with low PCV might allocate their energetic reserves in a different way to those fed on normal PCV blood. For example, if mosquitoes perceive the resource quality of their blood meal to be poor, they may re-allocate energetic reserves destined for long term survival to short-term reproduction (if long life seems unlikely). Consistent with the latter hypothesis, the apparent short-term fecundity benefit associated with low PCV blood in this investigation was offset by reduced long-term survival. Trade-offs between survival and reproduction are predicted to be most extreme when resource availability is low (Stearns & Stearns, 1992; Graves, 1993; Chapman & Partridge, 1996; Chapman *et al.*, 1998; Good & Tatar, 2001; Piper *et al.*, 2005). The

current findings showing an increase in short-term fecundity and concurrent decrease in long-term mosquito survival at low PCV might be potential evidence for such a trade-off.

An alternative explanation is that mosquitoes convert low PCV blood into egg resources more efficiently than normal blood, potentially because there are elements in blood serum (e.g. amino acids, lipids and other biochemical contents) that also contribute to egg production (Dimond *et al.*, 1956; Uchida *et al.*, 1990; Uchida *et al.*, 1998; Hansen *et al.*, 2004; Briegel *et al.*, 2002). So far there is little evidence that blood serum factors are important for triggering mosquito egg production (Hansen *et al.*, 2004). Regardless of the mechanism, blood protein is unlikely to provide a sufficiently reliable signal to drive mosquito fecundity.

7.2.2 Why were malaria parasites not virulent to their vectors

There was no evidence that the fitness of *An. gambiae* s.s. mosquitoes was impaired by consuming *P. falciparum*-infected blood, and going on to develop oocyst infections in current study. Here it is important to ask why would a parasite risk killing its vector if it compromised its ongoing transmission to new host? (Williams & Nesse, 1991). Virulence could be a mistake, or maladaptative outcome, that only happens when a parasite is in an abnormal and/or harsh environment (Levin & Bull, 1994). Alternatively, it has been hypothesized that some degree of virulence to vectors can be beneficial to parasite transmission (Frank, 1996), especially it improves parasite replication at the cost of a vector fitness trait with little direct impact on its transmission (e.g. reproduction rather than survival).

Many previous field studies of natural parasite-vector interactions are in agreement with the current results that malaria infection did not reduce mosquito survival (Robert *et al.*, 1990; Boyd, 1940; Chege & Beier, 1990; Gamage-Mendis *et al.*, 1993; Hogg & Hurd, 1997; Lines *et al.*, 1991). However, one previous field study suggested that the mortality of *An. gambiae* mosquitoes which carry sporozoites of *P. falciparum* is increased (Anderson *et al.*, 1999b). Possible hypotheses for why infected mosquitoes may have lower survival include that they have decreased efficiency in obtaining blood and thus have to probe hosts more often and be more exposed to their defensive behaviour (Rossignol *et al.*, 1984; Ribeiro, 2000). In contrast to the majority of field studies, most studies using

laboratory models have found a negative impact of *Plasmodium* on mosquito survival (Klein *et al.*, 1982; Klein *et al.*, 1982; Ferguson & Read, 2002a; Gad *et al.*, 1979; Hogg & Hurd, 1995a; Hogg & Hurd, 1995b; Dawes *et al.*, 2009; Anderson *et al.*, 1999a; Lyimo & Koella, 1992). Almost all of these studies have been conducted on unnatural vector-parasite combinations. Results gathered here further strengthen the hypothesis that *Plasmodium* is only virulent in vectors it has not co-evolved with, and infection does not decrease the survival of the natural mosquito vectors (Ferguson & Read, 2002b).

As virulence defined, there has been extensive study of the impact of *Plasmodium* on mosquito fecundity (Hurd, 2001; Ferguson *et al.*, 2003b; Hogg *et al.*, 1995; Hogg & Hurd, 1995a; Gray & Bradley, 2006; Dao *et al.*, 2010). Several studies of both laboratory models (Hacker & Kilama, 1974; Ferguson *et al.*, 2003b; Hurd, 2001; Gray & Bradley, 2006), and natural infections (Hogg *et al.*, 1995) have reported that malaria infection reduces mosquito fecundity. However, one study showed that in an area of malaria endemic transmission, *An gambiae* mosquitoes which were exposed to gametocytes in naturally-infected blood had much higher oviposition rate (around 6-fold) than those fed uninfected blood (Ferguson *et al.*, 2005). However in the latter experiments, mosquitoes never went onto develop oocysts so this change in reproduction was specifically due to exposure to, but not infection with, parasites. Mice with severe (*P. chabaudi*) infection were also observed to affect mosquito fecundity (Ferguson *et al.*, 2003a). Of the 'CR and ER' parasite genotypes investigated here, 'mixed genotype infections' were associated with 20% decrease in mosquito fecundity and 'single genotype (CR or ER genotype)' with no change in mosquito fecundity compare to uninfected controls (Ferguson *et al.*, 2003a). These studies suggested that there may some advantages of parasite-infected blood to vector fecundity that could partially compensate for negative effects due to parasitism. Possible causes of this effect could be changes in the amino acid composition of human blood that sometimes occur with malaria infection (Enwonwu *et al.*, 2000), or other unknown benefits of low PCV on mosquito oviposition.

Another possible hypothesis for why virulence was not observed in this study, but was in others, could be that the *in vitro* system used does not capture other haematological correlates of malaria-infected blood meals that are indirectly responsible for virulence. The *in vitro* membrane system used here cannot mimic all aspects of a live, malaria-infected host. Although the impact of PCV was specifically investigated here, malaria

infection in human induces several other haematological changes such as changes in blood biochemistry, presence of cytokines or other immune molecules (Tewari *et al.*, 2005; Sherman, 1991; Ramiro *et al.*, 2011; Raberg *et al.*, 2006; Gupta, 2005; Koella & Boete, 2003; Enwonwu *et al.*, 2000). There is currently limited or no information on the influence of these factors on mosquito fitness. If these factors have a negative impact on mosquito feeding, fecundity or survival, the experimental design used here could significantly underestimate the genuine virulence of human malaria parasites to their vectors under more natural settings.

7.3 Caveats

If the effects of PCV on mosquito infection rate occur in nature, PCV could influence malaria transmission. However this prediction has several caveats that would need to be tested. While, neither low red cell density nor *P. falciparum* infection were found to have a detrimental impact on mosquito vector fitness in this study, it is possible that the artificial nature of the *in vitro* system used may have underestimated the costliness of the factors tested to mosquitoes. Firstly, malaria infection in human induces several other haematological changes such as changes in blood biochemistry, presence of cytokines or other immune molecules (Tewari *et al.*, 2005; Sherman, 1991; Ramiro *et al.*, 2011; Raberg *et al.*, 2006; Gupta, 2005; Koella & Boete, 2003; Enwonwu *et al.*, 2000) other than low PCV in vertebrate hosts that might have consequences for mosquito vector fitness. If this is the case, parasite virulence could be underestimated with an *in vitro* system. Furthermore, all mosquitoes were maintained under ideal, standardized insectary conditions in which their diet was supplemented by *ad libitum* provision of glucose solution, which is known to enhance mosquito survival and energetic reserves (Foster, 1995; Straif & Beier, 1996; Gary & Foster, 2001; Rivero & Ferguson, 2003). Consequently even if the nutritional quality of low PCV and/or *P. falciparum*-infected blood meals is lower than those taken from uninfected control blood, this effect may not have been detected if mosquitoes could offset these effects by increasing their intake of sugar and/or minimizing their activity and energetic demands. To assess this, the experiments could be repeated under more natural conditions such as a semi-field system (Nghabi *et al.*, 2008). Additionally, further laboratory experimentation in which mosquitoes were subjected to more adverse conditions (e.g. glucose limitation, temperature extremes), could also be useful to assess this possibility.

Another limitation of this study was that in all experiments, mosquito fitness was assessed after taking only one blood meal. In nature, female *An. gambiae* s.s. will blood feed repeatedly every 2-4 days of their adult life (Romoser *et al.*, 1989; Briegel & Horler, 1993; Ramasamy *et al.*, 2000), with the median number of blood meals taken by mosquitoes in natural populations being two (Gillies & Wilkes, 1965). If the fitness costs of a 'poor quality' blood meal, whether due to reduced PCV or malaria parasitism, are cumulative and/or increase with mosquito age, they would not be detected in these experiments. One laboratory study found that when *An. gambiae s.s.* mosquitoes were fed on single blood meals from different animal species, some host species were associated with poorer mosquito fitness than others. However, when mosquitoes obtained two blood meals of any type, there was no evidence of any negative effects on vector fitness (Lyimo *et al.*, 2012). This evidence suggests that as long as mosquitoes obtain one 'good quality' blood meal there may be no longer lasting effect of having one 'bad quality' blood meal. The investigation of multiple feeds on different hosts with varying PCV would give a better representation of the impact of this haematological trait under more natural conditions.

Another caveat for the interpretation of these results is that during these experiments, the mosquito insectaries became contaminated with Microsporidia which reduced the number of mosquitoes available during the second year of this project. Previous studies on other invertebrates suggest that Microsporidia infection can increase the mortality of insects (Lorenz & Koella, 2011), and also may have other effects on invertebrate reproduction such as sex ratio distortion (e.g. *Corophium volutator*) (Mautner *et al.*, 2007; Haine *et al.*, 2004). Known effects of Microsporidia on *An. gambiae* s.s. mosquitoes include delayed pupation (by 10%), decreased fecundity (by 23%), reduced adult lifespan (by 27%) (Lorenz & Koella, 2011) and possibly reduced susceptibility to malaria infection (Bargielowski & Koella, 2009). Thus it is possible that Microsporidia infection influenced mosquito fitness parameters in the present study. However, as mosquitoes from the insectary were randomly assigned to different experimental treatments, it is unlikely that this could have caused a systematic bias in which the impact of PCV was confounded by Microsporidia. The data on infection with *P. falciparum* was from a period when Microsporidia infection was not known to be present. Even if there was some low level of Microsporidia during these infection experiments, it would again have been randomly

distributed throughout PCV treatments, and could not explain why infection rates were thus much higher in one PCV group than the other.

Ultimately, confirmation of these laboratory-based findings would require numerous experimental infections of *An. gambiae* s.s. from people representing a range of gametocyte density and PCV conditions in a malaria endemic setting, under more natural mosquito holding conditions. This approach poses numerous logistic and ethical challenges that will require the development of more appropriate methodology (e.g. more realistic mosquito containment systems). Until this becomes available, the experimental approach used here can provide a useful foundation for generating testable hypotheses and predictions of how host haematological factors could influence malaria transmission in nature.

7.4 Epidemiological and ecological implications of these results

Before the implications of these results can be extrapolated to field settings, other host factors that may change concurrently with anaemia and influence transmission need to be considered. It has been hypothesized that that hosts who are anaemic may have less effective anti-vector behaviour (Day & Edman, 1983; Ewald, 1994). Theoretical predictions that parasites should evolve to induce symptoms that enhance pathogen transmission (Frank, 1996; Ewald, 1994; Day, 2002b; Day, 2001; Paul *et al.*, 2004; Stearn & Koella, 2008) have received some empirical support from observations that mosquitoes have higher feeding success on malaria-infected, anaemic mice than on healthy controls (Day & Edman, 1983). To my knowledge there has not been any demonstration that anaemic humans are more attractive to malaria vectors. However if this phenomenon does occur in people, it could combine with the enhanced infectiousness of parasites in low PCV blood demonstrated here to generate a transmission advantage from anaemic hosts, even higher than ~50% estimated here. However, there are several other factors operating in natural settings that could reduce the transmission advantage of low PCV blood. Humans who become the most ill with severe anaemia due to infection may also be more likely to seek treatment and take drugs that kill gametocytes, than asymptomatic carriers. Further investigation into the infectiousness and treatment seeking behaviour of

malaria-infected people with variable degrees of anaemia are necessary to resolve these hypotheses.

In addition to malaria transmission, the results also have implications for understanding the contribution of host factors to the fitness and dynamics of mosquito vector populations. It is generally thought that external environmental factors (e.g. temperature, availability of aquatic breeding sites, (Claborn *et al.*, 2002; Delatte *et al.*, 2009; Kirby & Lindsay, 2009; Lyimo *et al.*, 1992; Reiskind & Lounibos, 2009) and larval nutrition (Timmermann & Briegel, 1993; Fillinger & Lindsay, 2011; Hancock & Foster, 1997; Kaufman *et al.*, 2006)) are the most important determinants of mosquito survival and reproduction in natural habitats. While access to blood is also known to be a primary determinant of mosquito reproduction, little is known about whether variation in the composition and quality of blood meals taken from hosts has a significant impact on *Anopheles* fitness. The reproductive success and survival of several mosquito species is known to be host species dependent (Lyimo & Ferguson, 2009; Lyimo *et al.*, 2012). However, little is known about the role of specific variation within a host species on the fitness of mosquitoes. This thesis provides two lines of evidence to suggest that haematological variation within humans may have some impact on *Anopheles* fitness. First of all, in pilot experiments (chapter 3), the (human) host individual from which blood was taken was found to have relatively minor (1-2% difference) but statistically significant effects on *An. gambiae* s.s. blood meal size. Secondly, the fecundity of mosquitoes after one blood meal was found to be significantly higher after feeding on blood of low rather than normal PCV (chapter 4). As discussed previously, the causes of the effect are not yet known and may be a result of energetic re-allocation on the part of mosquitoes rather than variation in blood meal quality. However it does suggest that *An. gambiae* s.s. obtain variable fitness rewards from feeding on different people, and thus that there is some potential for natural selection to generate a preference towards hosts with traits most beneficial to vector fitness. Field studies comparing the relative attractiveness of different people to mosquitoes, with the fitness benefits mosquitoes obtain from feeding on them, is needed to assess this possibility.

7.5 Conclusions and perspectives

At the start of this study, four hypotheses were proposed for how reductions in the Packed Cell Volume of human hosts, consistent with severe anaemia, could influence the transmission success of malaria parasites, by changing: (1) mosquito vector fitness, (2) parasite infectiousness to mosquitoes, (3) the sporogonic success of parasites to mosquitoes, and/or (4) the virulence that mosquito vectors experience from malaria infection. In most cases, the impact of reduced PCV on parasite and mosquito fitness was predicted to be negative. Few of these original predictions were met, in that reduced blood PCV had no impact on malaria sporogonic success or virulence to mosquitoes, and enhanced the infectiousness of parasites to mosquitoes and some aspects of their fitness (fecundity). Whether the effects detected here would also be manifested under natural transmission settings remains to be tested, but the underlying hypotheses gained are that haematological variations in host red cell density do influence the outcome of malaria-vector interactions, and reductions in this trait associated with anaemia may act to strengthen the transmission cycle.

Two general hypotheses can be drawn from these observations with wider implications for the potential evolution and epidemiology of this transmission system. The first is that malaria disease severity as manifested by a reduction in red cell density may provide a fitness benefit to malaria parasites, and thus act as a source of selection on disease virulence. Similar predictions have been drawn from numerous laboratory studies (Read *et al.*, 1999; MacKinnon & Read, 1999b; Ferguson *et al.*, 2003a; Reece *et al.*, 2005). However, other field-based studies have suggested that healthy, asymptomatic carriers are more likely to infect mosquito vectors than individuals with severe malaria symptoms reporting to medical facilities (Gouagna et al 2005). The enhanced infectiousness of asymptomatic carriers in this case could not be explained by higher gametocyte densities, thus more complex factors may be operating in natural systems that do not favour virulent parasites.

A second hypothesis can be drawn about the general consequences of anaemia in a host population, malaria-associated or otherwise, for the fitness and dynamics of malaria vector populations. Reductions in PCV due to anaemia are common in malaria-endemic settings in Africa, arising from malnutrition, poor maternal health, and infection by

numerous pathogens including malaria (Green *et al.*, 2011; Bukar *et al.*, 2008; Abdelrahim *et al.*, 2009; Gibson, 2006; Nacher, 2002). For example, 92.6% of pregnant women in Gombe, Nigeria were anaemic as a result of parasitic infections such as helminth worm infections and malaria (van den Broek *et al.*, 1998; Bukar *et al.*, 2008). If the effects of reduced PCV on mosquito and malaria parasite fitness described here operate in natural settings, it could be that a range of public health improvements that independently target anaemia by improving malnutrition, maternal health, and reducing exposure to worm infections may provide knock-on benefits for malaria control by decreasing both the average infectiousness of the human population and the reproductive potential of vector populations. Until the results gathered here can be validated under natural conditions, this hypothesis remains highly speculative. However it reinforces the value of general health and economic improvement strategies as a means to tackle specific disease problems such as malaria.

7.6 What are the next steps?

As described above, probably the biggest open question from results obtained here is whether these results accurately represent the malaria transmission consequences of low human host PCV in more natural settings. To evaluate this further, I recommend the following further experiments.

Experiment 1. Investigating the effects of host blood PCV under more realistic mosquito feeding conditions

Some results presented here, such as the enhanced fecundity of mosquitoes fed on low PCV blood, and the avirulence of malaria parasites, differ from previous studies. I would like to test how features of experimental design such as the number of blood meals and mosquito holding conditions could influence results. I would plan further experiments to test the impact of PCV over a wider range of red cell density, with a wider range of blood meals and where mosquitoes were kept in more 'stressful' environments (e.g no glucose provided).

Experiment 2. Monitoring the effect of anaemia on vector fitness under natural field-based condition

Overall, my research showed malaria parasites had no harmful effect on vector fitness using the parameters of fitness measured. Different outcomes may occur under natural conditions using various hosts with varying haematological backgrounds. I would like to further investigate how host PCV influenced mosquito fitness in a semi-field system, looking at a wider range of possible fitness traits such as dispersal ability, anti-predator avoidance, hatching rate and development success of eggs produced from different kinds of blood meals.

Experiment 3. Mosquito fecundity signals

Although removed from the current study, I would also be interested in creating a laboratory model system for detailed studies of how variation in blood meal composition influences mosquito fecundity. Specifically I would like to investigate how variation in the biochemical composition of host blood associated with a variety of physiological factors (malaria infection, malnutrition, immune responses and genetical disorders) could influence the fitness of the mosquito vectors that feed on it. If and how mosquitoes could re-allocate their reproduction in accordance with blood meal quality is unknown, and I would like to investigate what kind of signals could be triggered by host, parasite or vector factors to influence their reproductive cycle.

Appendices

8.1 Appendix 1- Details of the sample size calculations in Chapter 3.

All the sample size calculations were conducted to estimate the number of mosquitoes needed in each experimental treatment in order to be able to detect a statistically significant difference in the fitness traits that was proposed to measure. Specifically, sample size calculations were performed on the basis of the mosquito fitness trait that was assumed was likely to be the most variable: fecundity (number of eggs laid per individual), not in survival or oviposition which were also measured .

The number of mosquitoes required to detect a statistically significant difference in fecundity (number of eggs that laid per individual) was been calculated using the following formula:

$N = [t]^2 \times 2 \times \text{variance} / (\text{difference to be detected})^2$ (Van Emden, 2008; Van Emden, 2008; Van Emden, 2008)

For using this formula, at the first needed to define the critical t-values [t] that need to be exceeded to reject a null hypothesis of similar fecundity, the expected variance in mosquito fecundity under standard laboratory conditions, and the difference in fecundity I aimed to detect. These values were calculated as follows:

i. Critical t-values: In this experiment there were 2 experimental factors (blood donor and blood storage age), each with three levels. Thus the degrees of freedom in a statistical model aiming to test both these factors are:

$df_{Factor\ 1} \times df_{Factor\ 2} = df_{Total}F$

(3-1) x (3-1) = 4df

Assuming a two tailed t-test would be performed with a power of 90% and a significance level of $p<0.05$ applied to reject the null hypothesis, the following critical t-values must be exceeded.

$t_{p\ 0.05,\ 4}$ = 2.776 - values above this indicate that the null hypothesis can be reliably rejected with a 95% probability.

$t_{p\ 0.10,\ 4}$ = 2.132 - data producing t-values above this indicate we could correctly accept Ho with 90% probability of being right (90% power of the test).

ii. Expected variance in mosquito: Data obtained from colleagues working with *An. gambiae* s.s in our laboratory indicated the mean number of eggs laid per mosquito =60 (SE = 4.1, Variance = 31.8, I. Lyimo pers. Comm.). In my experiments the minimum difference in mosquito fecundity I aimed to detect between treatments was 10%, which is equivalent to 6 eggs assuming the above mean.

iii. Mosquito sample size: Using the formula below and a values calculated above, the number of mosquitoes per treatment needed to detect a 10% difference in mean fecundity was calculated as:

$$N = [t]^2 \times 2 \times \text{variance} / (\text{difference to be detected})^2$$

$$N = [2.776 + 2.132]^2 \times 2 \times 31.8 / (6)^2$$

$$N = 1532 / 36 = 42$$

iv. Mosquito rearing to meet sample sizes: In order to the meet the minimum sample sizes required for analysis of fecundity as detailed above, a sufficient number of pupae were needed from the insectary colony that would emerge into adult females, take a blood feed, and lay eggs afterwards. Previous studies in the group indicated that approximately 80% of female *An. gambiae* s.s. will feed when offered a blood meal from a membrane feeder, and those of those, approximately 80% will go on to lay eggs. Thus accounting for the proportion of females that do not feed or lay, a total of 45 blood-fed and egg-laying mosquitoes should be obtained from 70 females that are offered a blood meal. Thus a total of 630 females are required for this experiment (70 per replicates x 3 donor treatments x 3 blood storage treatments). As the sex ratio of Anopheles pupae in our insectary is approximately 50:50, an estimated 1260 pupae were required from the lab colony for each time point of experiment.

8.2 Appendix 2- Summary of data in Chapter 3.

DONOR	TIME BLOOD STORED (DAYS)	REPLICATE NUMBER	NUMBER OF MOSQUITOES OFFERED BLOOD MEAL	NUMBER OF MOSQUITOES SUCCESSFULLY FEEDING	% FED
A	7 day	1	70	70	100%
		2	70	70	100%
		3	70	70	100%
A	14 days	1	70	67	96%
		2	70	69	99%
		3	70	67	96%
A	21 days	1	70	70	100%
		2	70	68	97%
		3	70	66	94%
B	7 day	1	70	70	100%
		2	70	70	100%
		3	70	70	100%
B	14 days	1	70	66	94%
		2	70	70	100%
		3	70	65	93%
B	21 days	1	70	68	97%
		2	70	67	96%
		3	70	67	96%
C	7 day	1	70	70	100%
		2	70	70	100%
		3	70	70	100%
C	14 days	1	70	69	99%
		2	70	68	97%
		3	70	64	91%
C	21 days	1	70	66	94%
		2	70	69	99%
		3	70	66	94%

Table 8.1. Proportion of mosquitoes who feed in membrane feeding trails with blood of different donors and storage times.

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Donor	Replicate	Blood storage time (days)	Total No. Mosquitoes offered a blood meal	No. of mosquitoes fed	No. mosquitoes laying eggs	% laying eggs
A	1	7	70	70	47	67.1
A	2	7	70	70	41	58.6
A	3	7	70	70	47	67.1
A	1	14	70	67	33	49.3
A	2	14	70	69	42	60.9
A	3	14	70	67	39	58.2
A	1	21	70	70	28	40.0
A	2	21	70	68	45	66.2
A	3	21	70	66	31	47.0
B	1	7	70	70	28	40.0
B	2	7	70	70	43	61.4
B	3	7	70	70	44	62.9
B	1	14	70	66	24	36.4
B	2	14	70	70	46	65.7
B	3	14	70	65	20	30.8
B	1	21	70	68	37	54.4
B	2	21	70	67	20	29.9
B	3	21	70	67	26	38.8
C	1	7	70	70	41	58.6
C	2	7	70	70	48	68.6
C	3	7	70	70	39	55.7
C	1	14	70	69	34	49.3
C	2	14	70	68	39	57.4
C	3	14	70	64	20	31.3
C	1	21	70	66	28	42.4
C	2	21	70	69	24	34.8
C	3	21	70	66	24	36.4

Table 8.2. Proportion of mosquitoes laying eggs after feeding on blood from different donors and blood storage times.

Donor	Replicate	Blood storage time (days)	Mean no. eggs per mosquito	Range of eggs per mosquito	SE
A	1	7	66.9	7-125	4
A	2	7	63.42	10-127	3.7
A	3	7	65.22	6-148	4.6
A	1	14	59.76	6-121	5.5
A	2	14	50.35	14-109	3.3
A	3	14	52.43	7-109	3.7
A	1	21	60.33	26-131	5.5
A	2	21	59.94	6-136	4.3
A	3	21	68.16	10-140	6.2
B	1	7	73.73	12-144	5.1
B	2	7	65.98	6-142	4
B	3	7	74.34	7-134	4
B	1	14	55.41	6-102	5.2
B	2	14	63.12	15-146	4.4
B	3	14	60.32	9-128	6.4
B	1	21	53.02	8-133	3.9
B	2	21	55.91	6-135	5
B	3	21	43.09	4-116	4.6
C	1	7	66.69	13-147	4.9
C	2	7	52.23	10-105	3.2
C	3	7	63.6	13-134	3.7
C	1	14	54.34	12-109	4.5
C	2	14	54.74	12-129	4.8
C	3	14	59.8	14-114	5.1
C	1	21	57.06	10-120	4.9
C	2	21	57.28	5-120	5.1
C	3	21	57.63	6-110	5.7

Table 8.3. Average number of eggs laid by mosquitoes in experiments where they fed on blood of different donors and storage times.

DONOR	REPLICATE NUMBER	BLOOD STORAGE TIME (DAYS)	NUMBER OF MOSQUITOES ANALYSED FOR LONGEVITY	MEDIAN DAY OF DEATH POST-BLOODMEAL	RANGE OF DAY OF DEATH POST-BLOODMEAL
A	1	7	70	17.5	10-27
A	2	7	70	17	10-35
A	3	7	70	17	10-35
A	1	14	67	26	16-42
A	2	14	69	26	16-35
A	3	14	67	24	18-33
A	1	21	70	33	24-42
A	2	21	68	33	24-42
A	3	21	66	32	23-38
B	1	7	70	18	9-26
B	2	7	70	17.5	10-30
B	3	7	70	19	11-35
B	1	14	66	24	16-32
B	2	14	70	24	18-32
B	3	14	65	25	16-35
B	1	21	68	33	23-39
B	2	21	67	31	24-42
B	3	21	67	33	23-42
C	1	7	70	17	10-30
C	2	7	70	17	10-25
C	3	7	70	17	10-36
C	1	14	69	26	16-36
C	2	14	68	26	16-37
C	3	14	64	26	16-34
C	1	21	66	31	23-41
C	2	21	69	33	23-40
C	3	21	66	34	23-43

Table 8.4. Survival data of mosquitoes feeding on blood of different donor and storage times.

8.3 Appendix 3- Details of the sample size calculations in Chapter 4.

Sample size calculations were conducted to estimate the approximate number of mosquitoes required in each treatment group to detect a statistically significant difference in one of central mosquito fitness traits of interest: mosquito fecundity. The number of mosquitoes required to detect a difference in mosquito fecundity between treatments was calculated using the following formula (Van Emden, 2008):

$N = [t]^2 \times 2 \times \text{variance} / (\text{difference to be detected})^2$

In this experiment, there were 2 PCV treatments. Thus the degrees of freedom used in the analyses testing for differences between treatments were 2-1 = 1df. Sample size calculations were based on the number of individuals required to detect a difference in mosquito fecundity between the different groups using a 2 tailed t-test. Under standard laboratory conditions, the average fecundity of *An. gambiae* s.s. is 60 eggs/ mosquito (obtained from data of previous experiment chapter 3). The aim was to have sufficient power to detect a difference of at least 10% in mosquito fecundity between treatment groups (thus the magnitude of the difference to be detected was calculated as 60 x 0.10 = 6 eggs). To detect a statistically difference of this magnitude at *p* value =0.05 with a power of 90%, the following critical t-values must be exceeded.

$t_{(p\text{-value})\ 0.05,\ df\ 1} = 12.71$ – data producing t-values above this would indicate that the null hypothesis can be rejected with a 90% probability of being right.

$t_{(p\text{-value})\ 0.10,\ df\ 1} = 6.314$ – data producing t-values above this indicate we could correctly accept Ho with 90% probability of being right (the power of test).

For calculating the more accurate method, two t values detected. The t value for t at *P* value = 0.05 plus that for t at the required level of certainty

These critical values were incorporated into the sample size calculation equation along with an estimate of the expected variance in mosquito fecundity under typical conditions in our insectary, (variance =2.89 for mean fecundity of 60 eggs, data collected from pervious experiment based on approximately 630 mosquitoes). Using the formula below, estimates of the variance, and threshold t-values for my experimental design, I calculated the number of mosquitoes per treatment that would be needed to detect a 10% difference in mean fecundity:

$N = [t]^2 \times 2 \times \text{variance} / (\text{difference to be detected})^2$

$N = [6.314 + 12.71]^2 \times 2 \times 2.9 / (6)^2$

$N = 2166 / 36 = 60$

The calculation above shows that a sample size of 60 egg-laying female mosquitoes would be required in each treatment to detect a statistical difference of the desired magnitude (10%). Not all mosquitoes offered a blood meal actually feed, and not all that feed lay eggs. Thus in order to get a sample size of 60 egg laying females per treatment, the actual number of females to be offered a blood meal was increased to take into account that approximately 10% will not feed, and 10% of these will not lay eggs. The total sample size was also increase by a further 14% to offset losses due to death occurring between the time of the blood feed and the day on which mosquito fecundity can be assessed (4 days after feeding). Therefore to obtain a sample size of 91 egg laying mosquitoes, a total of 129 females were allocated to each treatment before the blood feed. This experiment was repeated seven times.

8.4 Appendix 4- Summary of data in Chapter 4.

PCV	REPLICATE	NO. MOSQUITOES OFFERED A BLOOD MEAL	NO. MOSQUITOES TAKING A BLOOD MEAL	MEAN BLOOD MEAL SIZE (UG)	SEM OF BLOOD MEAL SIZE	RANGE OF BLOOD MEAL SIZE (UG)
Normal	1	85	75	11.39	0.5	2.5-22.2
Normal	2	77	56	9.6	0.6	0.005-20.7
Normal	3	100	91	8.5	0.4	2.1-21.9
Normal	4	125	114	13.59	0.7	1.6-32.8
Normal	5	125	113	10.56	0.5	0.001-24.4
Normal	6	100	86	9.32	0.6	0.9-28.4
Normal	7	80	79	15.2	0.9	2.5-32
Low	1	80	60	4.0	0.2	1.8-9.8
Low	2	59	50	2.5	0.3	0-9.5
Low	3	110	96	3.7	0.1	1.8-10.1
Low	4	125	70	5.17	0.5	1.4-23.8
Low	5	125	103	5.15	0.4	0.008-18.99
Low	6	100	51	3.42	0.4	1.4-10.9
Low	7	75	36	3.8	0.6	1.4-16.8

Table 8.5. Mean blood meal size (haematin excreted) for seven replicates. In each experiment, mosquitoes were offered one of two treatments- normal red blood cell density and blood of low red blood cell density.

PCV	REPLICATE	NO. MOSQUITOES TAKING A BLOOD MEAL	NO. MOSQUITOES LAYING EGGS	OVIPOSITION RATE
Normal	1	75	9	0.12
Normal	2	56	28	0.50
Normal	3	91	30	0.33
Normal	4	114	28	0.25
Normal	5	113	46	0.41
Normal	6	86	7	0.08
Normal	7	79	20	0.25
Low	1	60	18	0.30
Low	2	50	24	0.48
Low	3	96	30	0.31
Low	4	70	36	0.51
Low	5	103	46	0.45
Low	6	51	2	0.04
Low	7	36	15	0.42

Table 8.6. Oviposition rates for mosquitoes feeding on blood of different packed cell volumes.

PCV	REPLICATE	NO. MOSQUITOES LAYING EGGS	MEAN EGGS	RANGE	SEM
Normal	1	9	54	19-88	6.2
Normal	2	28	46	9-113	4.5
Normal	3	30	45	3-95	4.2
Normal	4	28	43	5- 113	7.8
Normal	5	46	49	9- 94	5.8
Normal	6	7	41	5- 105	26.1
Normal	7	20	50	8- 97	9.2
Low	1	18	51	6-104	5.7
Low	2	24	54	15-90	3.9
Low	3	30	45	6-153	6.1
Low	4	36	62	7- 124	5.1
Low	5	46	70	10- 141	4.1
Low	6	2	35	8- 62	23.1
Low	7	15	61	6- 111	7.1

Table 8.7. Mean, SEM and range of eggs laid by mosquitoes feeding on blood of different PCV.

8.5 Appendix 5- Details of the sample size calculations in Chapter 6.

Sample size calculations were conducted to estimate the approximate number of mosquitoes required in each treatment group to detect a statistically significant difference in one of central mosquito fitness traits of interest: mosquito fecundity.

As in the chapter 4, the number of mosquitoes required to detect a difference in mosquito fecundity between treatments was calculated using the following formula:

$N = [t]^2 \times 2 \times \text{variance} / (\text{difference to be detected})^2$ (Van Emden, 2008)

In this experiment, there were 2 PCV treatments and 2 parasite treatments. Thus the degrees of freedom used in the analyses testing for differences between treatments were e.g. degree of freedom associated with PCV= 2-1 = 1df, degree of freedom associated with parasite treatment = 2-1= 1df, degree of freedom associated with PCV * parasite treatment = 1df * 1df = 1df.

Sample size calculations were based on the number of individuals required to detect a difference in mosquito fecundity between the different groups using a 2 tailed t-test. Under standard laboratory condition, the average fecundity of *An. gambiae* s.s. is 60 eggs/ mosquito (obtained from data of previous experiment with *An. gambiae* s.s, chapter 3). A detectable difference of at least 10% in fecundity between treatment groups (10% difference from assumed average of 60 eggs = difference of 6 eggs) was decided upon.

The aim was to have sufficient power to detect a difference of at least 10% in mosquito fecundity between treatment groups (thus the magnitude of the difference to be detected was calculated as 60 x 0.10 = 6 eggs). To detect a statistically difference of this magnitude at *p* value =0.05 with a power of 90%, the following critical t-values must be exceeded. To detect a statistically difference between these treatments groups at p=0.05, with a power of 90%, the following critical t-values must be exceeded. The critical t value is obtained from the critical region of a hypothesis test. It leads to the decision that there is a difference between treatments groups and the null hypothesis to be rejected in favor of the alternative hypothesis.

$t_{(p\text{-value}) 0.05, df 1} = 12.71$ – data producing t-values above this would indicate that the null hypothesis can be rejected with a 90% probability of being right.

$t_{(p\text{-value}) 0.10, df 1} = 6.314$ – data producing t-values above this indicate we could correctly accept Ho with 90% probability of being right (the power of test).

These critical values were incorporated into the sample size calculation equation along with an estimate of the expected variance in mosquito fecundity under typical conditions in our insectary, (obtained from data of previous experiment with *An. gambiae* s.s in chapter 1). In previous work, the mean number of eggs laid by mosquitoes was 60, with a standard deviation of 1.7 (based on approximately 630 mosquitoes) and a variance of 3. The minimum difference to be detected in the number of eggs was set at 10%, which with a mean of 60 eggs, gave a difference of 6 eggs.

Using the formula below, estimates of the variance, and threshold t-values for my experimental design, I calculated the number of mosquitoes per treatment that would be needed to detect a 10% difference in mean fecundity:

$N = [t]^2 \times 2 \times \text{variance} / (\text{difference to be detected})^2$

$N = [6.314 + 12.71]^2 \times 2 \times 3 / (6)^2$

N= 2166/ 36= 60

The calculation above shows that this would result in a sample size of 60 egg-laying female mosquitoes. This sample size would be required in each treatment to detect a statistical difference of the desired magnitude (10%).

Not all mosquitoes offered a blood meal actually feed, and not all that feed lay eggs. Thus in order to get a sample size of 60 eggs batches per treatment, the actual number of females to be offered a blood meal was increased to take into account that approximately 10% will not feed, and 40% of these will not lay eggs. The total sample size was also increase by a further 14% to offset losses due to death occurring between the time of the feed and the day in which mosquitoes are dissected to assess whether they has become infected (day 10 after blood feeding). Dissections on day ten post blood feed are carried out to test for a difference in parasite infection rate.

Therefore, to obtain a sample size of 60 mosquitoes whose fecundity and infection status can be assayed, a total of 129 females were allocated to each treatment before the blood feed. This number of mosquitoes should result in 116 blood fed mosquitoes, 70 of which should lay eggs, and 60 of these should still be alive ten days after the blood meal. Thus as there were 4 treatment groups in the experiment, approximately 520 (4 x 129) adult female mosquitoes were required to run one experimental replicate. As the sex ratio of *Anopheles* pupae in our insectary is approximately 50:50, approximately 1040 pupae were required from the lab colony for each replicate. This experiment was repeated seven times with each replicate 2 weeks apart over six weeks.

8.6 Appendix 6- Summary of data in Chapter 5-6.

Replicate	Blood Packed Cell Volume (PCV)	No.dissected mosquitoes	No. infected mosquitoes	Infection rate (%)	Median oocyst intensity in infected mosquitoes
1	Normal (50%)	46	9	19.6 ± 0.04	3
	Low (15%)	50	23	46.0 ± 0.04	5
2	Normal (50%)	35	11	30.6 ± 0.05	3
	Low (15%)	34	11	31.4 ± 0.05	7
3	Normal (50%)	24	11	48.0 ± 0.06	16
	Low (15%)	25	19	86.0 ± 0.07	5

Table 8.8. Parasite infection results for mosquitoes offered gametocyte-infected normal blood (PCV= 40-50%) and low PCV blood (PCV= 10-15%). n.a. = not applicable (no infected mosquitoes).

REPLICATE	BLOOD FEED	NO. MOSQUITOES OFFERED A BLOOD MEAL	NO. MOSQUITOES TAKING A BLOOD MEAL	MEAN BLOOD MEAL SIZE (UG/ML)	SEM	RANGE OF BLOOD MEAL SIZE
1	Infected +Normal	105	81	8.9	0.4	1.5-20.7
	Infected +Anaemic	106	95	5.5	0.2	2.1-10.6
1	Uninfected +Normal	85	75	11.4	0.5	2.5-22.2
	Uninfected +Anaemic	80	60	4.0	0.2	1.8-9.8
2	Infected +Normal	85	75	11.4	0.5	2.5-22.2
	Infected +Anaemic	80	60	4.0	0.2	1.8-9.8
2	Uninfected +Normal	77	56	9.6	0.6	0.005-20.7
	Uninfected +Anaemic	59	50	2.5	0.3	0-9.5
3	Infected +Normal	125	116	13.3	0.6	1.8-32.3
	Infected +Anaemic	125	89	4.9	0.4	1.4-12.4
3	Uninfected +Normal	125	114	13.59	0.7	1.6-32.8
	Uninfected +Anaemic	125	70	5.17	0.5	1.4-23.8

Table 8.9. Mean blood meal size (haematin excreted) for seven replicates. In each experiment, mosquitoes were offered one of four treatments – normal blood with and without *P. falciparum* gametocytes, and blood of low PCV (anaemic) with and without gametocytes. Infection of mosquitoes with parasites occurred in four replicates.

REPLICATE	BLOOD FEED	NO. MOSQUITOES TAKING A BLOOD MEAL	NO. MOSQUITOES LAYING EGGS	OVIPOSITION RATE
1	Infected +Normal	81	45	0.56
	Infected +Anaemic	95	49	0.52
1	Uninfected +Normal	75	9	0.12
	Uninfected +Anaemic	60	18	0.3
2	Infected +Normal	75	29	0.39
	Infected +Anaemic	60	32	0.53
2	Uninfected +Normal	56	28	0.5
	Uninfected +Anaemic	50	24	0.48
3	Infected +Normal	116	43	0.37
	Infected +Anaemic	89	46	0.52
3	Uninfected +Normal	114	28	0.25
	Uninfected +Anaemic	70	36	0.51

Table 8.10. Oviposition rates for mosquitoes feeding on gametocyte-infected and non-infected blood of different PCV (normal and low).

REPLICATE	BLOOD FEED	NO. MOSQUITOES LAYING EGGS	MEAN EGGS	RANGE	SEM
1	Infected +Normal	45	53	7-150	4.5
	Infected +Anaemic	49	55	5-153	4.7
1	Uninfected +Normal	9	54	19-88	6.2
	Uninfected +Anaemic	18	51	6-104	5.7
2	Infected +Normal	29	40	8-115	5.0
	Infected +Anaemic	32	58	16-112	4.8
2	Uninfected +Normal	28	46	9-113	4.5
	Uninfected +Anaemic	24	54	15-90	3.9
3	Infected +Normal	43	50	5-130	6.8
	Infected +Anaemic	46	67	5-163	4.7
3	Uninfected +Normal	28	43	5-113	7.8
	Uninfected +Anaemic	36	62	7-124	5.1

Table 8.11. Mean, standard error and range of eggs laid by mosquitoes feeding on gametocyte-infected and non-infected blood of different PCV (normal and low).

8.7 Appendix 7- Summary results of Chapter 6.

Response variables	Interaction	P value	The best model	Separated groups	Fixed variables effects	P value
Bloodmeal size	PCV * blood infectious status	√ p<0.001	Model with interaction	Normal PCV	U.B.> I.B.	P<0.001
					Wing (+)	P<0.001
				Low PCV	U.B. = I.B.	p= 0.66
					Wing (+)	P<0.001
Bloodmeal size	PCV * oocyst infection rate	√ p=0.03	Model with interaction	Normal PCV	O(+)=O(-)	P= 0.49
					Wing (+)	P<0.001
				Low PCV	O(+)=O(-)	P= 0.59
					Wing (+)	P<0.001
Oviposition rate	PCV * blood infectious status	× p=0.44	Wing + blood PCV+ blood infectious status : p= 0.004	I.B. low PCV > U.B. low PCV>I.B. normal PCV>U.B. normal PCV Wing (+)		
Oviposition rate	PCV * oocyst infection rate	× p= 0.11	Wing + oocyst infection rate: p<0.001	O(+) > O(-)		
				Wing (+)		
			Blood PCV: p= 0.88			
Fecundity	PCV * blood infectious status	× p= 0.78	Blood PCV + wing : p<0.001	PCV (N) <PCV (L)		
				Wing (+)		
			Blood infectious status : p= 0.54			
Fecundity	PCV * oocyst infection rate	× p= 0.96	Wing: p<0.001 (+)			
			Blood PCV: p= 0.16			
			Oocyst infection rate: p=0.21			
Survival until day 10	PCV * blood infectious status	√ p=0.04	Model with interaction	Normal PCV	U.B. = I.B.	P= 0.42
					Wing (+)	P= 0.02
				Low PCV	U.B. < I.B.	**P=0.050**
					Wing	**P=0.055**
Survival until day 10	PCV * blood infectious status + ovipostion status (Y/N)	√ p=0.01	Model with interaction	Normal PCV	U.B. = I.B.	P= 0.41
					Wing (+)	P= 0.01
					Laid E. >non-laid E.	P<0.001
				Low PCV	U.B. < I.B.	**P=0.050**
					Wing	**P=0.055**
					Laid E. >non-laid E.	P<0.001

Table 8.12. Summary results of Chapter 6 which are started from interaction between two fixed variables (Blood PCV, blood infectious status). The Backward model selection procedure was conducted in R using LRTs to test if their addition significantly improved the explanatory power of the model. The best model was collected by comparison of their log likelihood from these competitive models.

***: Interaction between two variables is tested.**

√: Significant interaction was observed.

×: Non-significant interaction was observed.

>: higher estimation in comparison to other group

<: lower estimation in comparison to other group

U.B.= Uninfected blood fed mosquitoes, I.B.=gametocyte infected blood fed mosquitoes, O(+)= mosquito with oocyst infection, O(-)= mosquitoes without oocyst infection, Wing(+)=positive correlation with mosquito wing length, PCV(N)= normal PCV blood fed mosquitoes, PCV(L)= low PCV blood fed mosquitoes.

Mosquito body resource measurements at natural day of death				
Mosquito group	Response variable	Explanatory variables	Fixed variables effects	P value
Uninfected blood fed mosquitoes	Lipid	Blood PCV	PCV (N) > PCV	P= 0.02
		wing		P=0.48
	Glucose	Blood PCV		**P= 0.055**
		wing		P= 0.17
	Glycogen	Blood PCV		P= 0.23
		wing		P= 0.08
Mosquito body resource measurements at day 10 after blood feeding				
Mosquito group	Response variable	Explanatory variables	Fixed variables effects	P value
Infected blood fed mosquitoes	Lipid	Blood PCV		P=0.24
		wing	(+) correlation	0.01
	Glucose	Blood PCV		P=0.06
		wing		**P=0.051**
	Glycogen	Blood PCV	PCV (N) > PCV (L)	P=0.04
		wing		P=0.18

Table 8.13. Summary result of mosquito body resource measurements in two separate group of uninfected and gametocyte infected blood fed mosquitoes. Wing (+)=positive correlation with mosquito wing length, PCV(N)= normal PCV blood fed mosquitoes, PCV(L)= low PCV blood fed mosquitoes.

>: higher estimation in comparison to other group

<: lower estimation in comparison to other group

8.8 Appendix 8- List of Accompanying Media and Solutions

Thawing Solution I: It is 12% (w/v) NaCl (BDH Laboratory Supplies, Cat. No. 102415K), in ddH_2O and 0.22 µm sterile- filtered; kept at 4°C.

Thawing Solution II: It is 1.6 % (w/v) NaCl (BDH Laboratory Supplies, Cat. No. 102415K), in ddH_2O and 0.22 µm sterile- filtered; kept at 4°C.

Thawing Solution III: It is 0.9 % (w/v) NaCl (BDH Laboratory Supplies, Cat. No. 102415K), in ddH_2O; 0.2% (w/v) dextrose and 0.22 µm sterile- filtered; kept at 4°C.

Freezing Solution: It contains 0.65% (w/v) NaCl (BDH Laboratory Supplies, Cat. No. 102415K); 3.0% (w/v) D- sorbitol (Koch Light Laboratories Ltd, Cat. No. 5063-00); 28.0% (v/v) glycerol (BDH Laboratory Supplies, Cat. No. 101186M), in ddH_2O and 0.22 µm sterile-filtered; kept at 4°C.

Incomplete RPMI 1640 medium: 10.4 g/l GIBCO ® RMPI 1640 (+ L-glutamine, -NaHCO3) (Invitrogen, Cat. No. 51800019); 5.94 g/l (25mM) 1-piperazineethane sulfonic acid (HEPES) (Sigma, Cat. No. H3375); 50mg/l (0.37 mM) hypoxanthine (Sigma, Cat. No. H9636); in ddH2O, adjusted to pH 7.2; 0.22 µm sterile- filtered and utilised within four weeks; kept at 4°C.

Complete RPMI 1640 medium: 500 ml incomplete RPMI 1640 medium plus 21 ml of sterile- filtered 5% (w/v) NaHCO3 (Fisher Scientific, Cat. No. S/4240/60) in ddH2O; utilised within 1 week; kept at 4°C; 50 ml (10% v/v) sterile heat- inactivated (1hour at 56 °C) pooled human AB serum; kept at -75°C.

Giemsa's buffer solution: 3.0 g/l (21.1 mM) Na_2HPO_4 (BDH Laboratory Supplies, Cat. No. 102494C); 0.6 g/l (4.4 mM) KH_2PO_4 (Fisher Scientific Ltd, Cat. No. P/4800/53); in ddH_2O, adjusted to pH 7.2-7.4 and kept at room temperature.

Giemsa's stain: 5% (v/v) Giemsa's stain solution, Gurr ® improved R66 (BDH Laboratory Supplies, Cat. No. 350864); 95% (v/v) Giemsa's buffer solution, which was used for staining methanol fixed blood smears for 35 mins for asexual parasites and 40 min sexual parasites, then rinse slides in H2O. Daily fresh Giemsa's stain made.

Glucose solution for adult mosquitoes: 5.0% (w/v) D-glucose (Fisher Scientific Ltd, Cat. No. P/0500/53); 0.05% (w/v) para-amino-benzoic acid (PABA) (Fisher Scientific Ltd, Cat. No. P/2840/48), in ddH_2O and kept at 4°C.

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