The evolutionary ecology of parasite strategies for within-host survival

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Abstract

Plasmodium parasites, the causal agents of malaria, engage in complex interactions with their hosts, however despite decades of research much of their life cycle remains unexplored. A deeper understanding of the strategies parasite have evolved to survive within, and exploit hosts, offers novel approaches for treating infections. By integrating tools from different fields within parasitology with an eco-evolutionary framework, I explore some of the strategies *Plasmodium chabaudi* parasites deploy within poorly studied aspects of their life cycle. Using this rodent malaria model, I first tested the relationship between host daily rhythms and the transition of parasites from developing in the liver to replication in the blood. In contrast to expectation, host circadian rhythms (i.e. feeding-fasting rhythms) do not influence the timing or the manner by which parasites begin the blood stage of their lifecycle. Moreover, how parasites undertake this critical step appears selectively neutral, suggesting that the rhythmicity in blood stage replication that is well-known in malaria parasites is rapidly established once in the blood. I then explored the ecology of sequestration (withdrawal from the blood to organs), a parasite strategy assumed to facilitate immune evasion and that is related to the manifestation of severe disease phenotypes, and potentially transmission. Specifically, I tested whether sequestration is scheduled to align with host rhythms driven by feeding-fasting or by photoperiod. I found little evidence for host rhythms affecting sequestration, or its consequences for replication. However, whether or not hosts experience disruption to their own rhythms influences sequestration in the lungs. Finally, I resolved controversy and conflicting reports concerning the role of innate immune responses on infection dynamics, especially during the establishment of infections. My comprehensive meta-analysis show that innate immune factors have only a minor impact on parasite replication in the blood. Overall, my thesis contributes to malaria knowledge by uncovering new aspects of parasite ecology and interactions with the host.

Lay Summary

The parasites that cause malaria have a complex life cycle that includes multiple hosts (mosquitoes and mammals) and multiple developmental transitions (liver, blood, and sexual stages). Within each host and throughout their development parasites are faced with unique environments and to survive parasites utilise different strategies to overcome the challenges associated with these environments. My thesis explored the strategies parasites employ to overcome challenges imposed by host daily rhythms. I show that parasite strategies that work for one stage of their life cycle may not be effective for a different parasite stage. I demonstrate that host daily schedules are not important to parasites when they emerge from the liver. I also examine if parasites that withdraw from circulation into other tissues at a specific time (a 'hiding' strategy) benefit by coordinating their withdrawal with host physiological schedules (i.e. the opportunities for parasites to hide changes throughout a day) or benefit parasites by allowing them to avoid specific hazards present in circulation. I show that parasites 'hide' differently depending on the host's daily schedule they encounter, however, I did not detect any benefits parasites gain from the different hiding patterns. Finally, I quantify the degree to which innate immune responses affect parasite growth, and generalize the overall impact of innate immunity across different rodent malaria parasite genotypes and species. I find that the effects of innate immune interventions are moderate but the misuse of parasitaemia (proportion of infected red blood cells) instead of counts prevents interpreting the direction of these effects (i.e. is the effect mediated by immunity or simply resource limitation?). Thus, I suggest authors overcome this issue by implementing parasite densities instead. Overall, my results highlight that considering some of the neglected stages of malaria parasites may challenge our current state of knowledge, however this is necessary to implement effective control/eradication programmes. My thesis is the culmination of interdisciplinary research that combines ecology, immunology and chronobiology and aims to illustrate how such an interdisciplinary approach is necessary for the understanding of complex processes such as the host-parasite interactions of malaria parasites.

Declaration

I declare that the work presented in this thesis has been conducted by me under the supervision of Professor Sarah Reece. Except where explicitly noted below, the work presented in this thesis is my own.

Chapter 2 - Sarah and I designed the experiment. Aidan and Petra helped with experimental techniques and advised on statistical analysis.
Chapter 3 - I carried out the experiment and analysed all data. Aidan and Aliz helped with the injections and Petra advised on the molecular techniques. Honours student, Peter Xu, helped with the data collection.
Chapter 4 - The data collection and analysis were conducted by myself under the guidance of Tsukushi Kamiya. Sarah and Philip helped with the immune classification and their interpretation.

I can confirm that the work presented in this thesis has not been submitted for any other degree or professional qualification.

Alejandra Herbert Mainero, September 2022

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Chapter 1. General Introduction

1. Evolutionary ecology in parasitology

Parasitism is one of the most fundamental ecological interactions and is widespread across the tree of life. Evolutionary ecology aims to investigate how and why interactions between organisms and their environment (i.e. ecology) select for the evolution of particular traits, phenotypes, and strategies. Thus, when applied to parasites, this approach explains the evolution of traits, phenotypes and strategies that underpin parasites' ability to survive within hosts and transmit between hosts (Schmid-Hempel, 2021). Here, we interchangeably refer to the traits, phenotypes, behaviours, decisions, and strategies exhibited by parasites. Similarly, throughout this thesis, we interchangeably use the terms parasites and pathogens.

Just as for traits in multicellular, free-living, organisms, traits can converge such that natural selection finds similar solutions to analogous problems across divergent parasite taxa. For example, the ability to feminise the host is found in multiple vertically transmitted parasites (i.e. transmission only occurs from a mother to her offspring) infecting multiple arthropod host taxa (Ironside and Alexander, 2015). Evasion of host immune defences is clearly important to pathogens and a diverse array of strategies enable this. For example, mimicking molecules involved in host effector and regulatory pathways, has evolved in *Legionella* who use their LegK1 protein to activate the host's *Nuclear factor kappa B (*NF-κB) pathway by mimicking its inhibitor (inhibitor of nuclear factor kappa, IκB*))*(Mondino et al., 2020). This might help prevent the death of macrophages (Ge et al., 2009) which is the host cell for *Legionella.* Death of the host cell is detrimental to the bacteria and so, strategies to delay host cell death are favoured by natural selection. Another evasion strategy used by parasites is to hide, some parasites go into places where the immune system is unlikely to find them; for example, the eyes as illustrated by the eye flukes (*Diplostoma spathaceum)*(Schmid-Hempel 2021). Similarly to hiding, pathogens sometimes evade immune responses by adopting an inactive form to maintain a low profile, persisting as quiescent forms,

for example, several virus become latent e.g. Herpes simplex virus (Schmid-Hempel 2021).

Whilst immune defences are a common challenge across parasite taxa, other parasite challenges are unique and so parasite strategies are tailored so closely to their host that they become highly specialized. This results in relationships like those between cuckoo birds and their hosts, by which the cuckoo's parasitic eggs visually mimic those of their host in multiple traits (Attard et al., 2017). The survival of cuckoos' eggs in the parasitized host's nest is dependent on the host not recognizing cuckoos' eggs as their own. Indeed, instances of host manipulation by parasites are thought to require highly specialised strategies built on intimate host-parasite relationships. For example, being able to alter the time of day that hosts undertake certain behaviours can benefit parasites and such 'global' control of the host is likely to be specific to individual host-parasite relationships (Westwood et al., 2019).

While abilities to evade immune defences and control host behaviour are intuitively beneficial (i.e. 'adaptive') to parasites, it is often unclear why parasites do certain things. An evolutionary approach provides a predictive framework to test the adaptive value (i.e. fitness costs/benefits) of parasite strategies, as well as explaining the origin and maintenance of parasite traits. Such understanding can be harnessed to predict the evolutionary responses of parasites to interventions designed to control infections. This is useful because knowledge of parasite strategies informs how to deploy interventions in a manner robust to parasite counter-evolution as well as revealing novel opportunities to develop interventions. In this chapter, I outline the key concepts from evolutionary ecology and apply them to malaria parasites, especially in the context of the aspects of their life cycle that form the focus of my thesis.

1.1 Life history theory

Life history, as the words imply, encompasses the activities an individual organism undertakes from birth until death (Schmid-Hempel, 2021). A classic example of a life history is at what size should an organism stop investing in its growth to begin investment into reproduction? The allocation of finite resources between competing

activities – such as between growth and reproduction – represents a resource allocation trade-off. Different ecologies and lifestyles select for different strategies to resolve trade-offs (Poulin, 2011). Resource allocation trade-offs exist with components of fitness too; for example, once resources are allocated to reproduction they are further divided between components such as the quantity and quality of offspring. A general rule is that fast-paced organisms reproduce earlier and have lots of offspring, spending little time and effort in rearing them, whereas slower-paced organisms, like humans, reproduce relatively later, produce fewer offspring, and invest highly in parental care (Stearns, 1976).

Sexually reproducing parasites also experience resource allocation trade-offs between growth and reproduction. Growth for some parasites – such as gastrointestinal nematodes - is simply an increase in body size, whereas for microparasites growth manifests as asexual within-host replication. Reproduction translates to transmission, with the number of new infections representing parasite "offspring" and can often involve a round of sexual reproduction. Parasites appear to follow the general rules that have been identified during decades of research on free-living multicellular taxa (Poulin, 2011; Schmid-Hempel, 2021). For example, the bigger gastrointestinal nematodes grow, the more eggs they can produce, but they cannot afford the time required to grow to a large size if infecting a host mounting a strong immune response, and so become sexually mature at a younger age that is less fecund (Gemmill, et al., 1999).

Applying life history theory to model parasite trade-offs from the start of infection until clearance or end of infection (Schmid-Hempel, 2021), helps to explain why parasites do things the way they do. For example, some within-host conditions may be optimal for parasite growth but suboptimal for their reproduction, and vice versa. A holistic examination of life history traits in parasites with complex life cycles comes with complications since multiple environments (i.e. within definitive/intermediate/vector hosts and their abiotic environments) can generate multi-scale links between parasite traits and fitness, depending on which environment they are expressed in. For example, at the start of infection in a naïve host, the immune response might not be damaging to the parasite, thus parasite growth is optimal because there are abundant resources and no dangers. Thus, investing in reproduction for species like

platyhelminths (Viney and Cable, 2011) at this point may be suboptimal because this comes at the expense of growth. Furthermore, activities in one part of the life cycle might impose constraints on what parasites should do in other life cycle stages. For example, the trematode *Coitocaecum parvum* maximises fitness when it can infect hosts throughout its full life cycle (Lagrue and Poulin, 2007). Specifically, the intermediate host *Paracalliope fluviatilis* provides resources for growth which enables maximal fecundity during sexual reproduction in the next host, the fish *Gobiomorphus cotidianus*. However, if the definitive fish host is not available in the environment, the life cycle must be abbreviated and sexual reproduction undertaken at a smaller size in the intermediate host. This is not ideal but better than no reproduction (Lagrue and Poulin, 2007). Thus, studies of resource allocation trade-offs and other strategies that are exhibited in certain phases of a complex cycle should be undertaken in the context of the causes and consequences of the full life cycle. An additional complication can arise when the selective drivers of a trait differ throughout its evolutionary history. Put another way, the reason a trait arose in the first place (its origin) may no longer be the reason that selection maintains it in the population. For example, parasitic catfish species *Trichomycterid*, use modified opercular teeth to anchor to their swimming hosts, yet the basal function of these teeth is for attaching to substrate in high flow conditions (river) where other non-parasitic catfish taxa live (Leung and Le Comber, 2014). Thus, attachment to substrate selected for the evolution of teeth but now, parasitism is the selective force that maintains this trait.

1.2 Adaptive phenotypic plasticity

The evolution of resource allocation trade-offs might be underpinned by different genetic components that determine each allocation pattern. Such traits are referred to as being (genetically) ‘fixed’ because a different genotype is required to produce a different phenotype. Thus, for an organism to alter its strategy, mutation of gene sequences must occur. In contrast, phenotypic plasticity provides flexible phenotypes from the same underlying genotype, that depend on external cues for each manifestation (Pfennig and West-Eberhard, 2021). When plastic responses to cues help to maintain fitness across different environmental conditions it is referred to as “adaptive phenotypic plasticity” (APP) (Pfennig and West-Eberhard, 2021). This can

be illustrated by the density-dependent behaviour of some bacteriophage viruses that alternate between lytic and non-lytic forms in *E. coli* (Duddy and Bassleri, 2021). These changes between lytic or lysogenic forms - in response to host cell density - enhance the probability of virus dissemination and thus the ability to infect new host cells (lysis occurs when at high cell density).

In general, APP helps the parasite cope with multiple challenges faced throughout their life cycle. This includes adjusting strategies to cope with how within-host conditions change during each infection and rapidly adopting different phenotypes to best exploit the opportunities of different hosts encountered during the life cycle (Reece et al., 2009). Interest has recently increased in how APP could help parasites cope with environmental changes that take place on time scales as short as a day, i.e. daily rhythms of host, vector, and the abiotic environment (Reece et al., 2017). For example, *Schistosoma* schedule the shedding of cercariae to maximise transmission opportunities (i.e. encounter rate) to their definitive host; when the definitive host is nocturnal, shedding occurs mostly during the late afternoon; whereas shedding occurs before midday when the next host is diurnal (Lu et al., 2009).

APP requires that environmental variation is at least in part, predictable by a reliable cue that parasites can detect and respond to (Pigliucci, 2001). Parasites may use the environmental change itself, in which case they are detecting and responding to the selective driver for altering phenotype, or they may respond to a proxy for environmental change. For example, *Schistosoma* within a snail host may be able to use host circadian clocks (or even their own) as a time signal to start/end shedding, or they may respond to a proxy cue that relate to time of day such as temperature. The use of a proxy introduces the risk for errors if the proxy is not a fully accurate indicator for environmental change. On the other hand, if the proxy signals impending environmental change, organisms have an advantage from preparing in advance (Vaze and Sharma, 2013).

Not all plastic phenotypes are adaptive (i.e., do not enhance fitness) and it is challenging to determine whether a change in a parasite phenotype is due to it mounting a plastic response, or whether environmental change has imposed or released a constraint that alters the parasite phenotype in a manner outside the

parasites' control (Schneider and Reece, 2021). Often, a parasite's phenotype is a product of both its own activities and the direct impact of its environment. For example, some gastric parasites extract nutrients from the host's food intake. The balance of nutrients depends on what the host eats and when, and so the host governs opportunities for the parasite to eat, but what the parasite can use will also depend on the parasite's metabolism (e.g. the range of enzymes they have to metabolize/store nutrients).

1.3 Contrasting APP vs fixed genetic traits

Contrasting APP against fixed traits can be very informative because they can act on different time scales. For example, APP can act within individual parasites, or the same generation, enabling quicker responses to environmental change than can occur through changes in the frequency of genes selected via microevolution. The capacity for APP to provide an appropriate and rapid response to environmental change make it seem intuitively superior to fixed trait evolution. However, APP is predicted to be limited by costs and constraints that fixed traits are free from (DeWitt et al., 1998). This includes APP requiring environmental sensing and response machinery that is costly to maintain and use, the risk of interpreting cues for environmental change incorrectly and mounting a fitness-reducing response, and the scope for APP to alter traits is expected to be narrower than can be achieved via microevolution (DeWitt et al., 1998). Organisms relying on fixed traits do not have to pay the costs, or take the risks, of environmental sensing because they are selected to adopt the best strategy according to the conditions they are most likely to encounter.

APP and fixed strategies are not mutually exclusive, APP itself evolves because it has a heritable genetic basis. This is illustrated by considering a reaction norm approach (Pigliucci, 2001), defined as the repertoire of phenotypes produced by a genotype across a range of environments (Figure 1.1). In this example, a hypothetical population of a parasite nematode species consists of 2 genotypes. They both mount plastic responses, by adopting different ages at sexual maturity depending on the nutritional conditions they experience. Their ages at maturity in a particular condition differ from each other demonstrating genetic variation (i.e. genetically hard-wired differences; the

lines have different intercepts on the y-axis). Furthermore, the patterns they exhibit across environments differ (i.e. the lines have different slopes), demonstrating a genotype-by-environment interaction (GxE). Quantifying the patterns of reaction norms and establishing the contributions of genetic differences is important because genetic variation (including GxE) is the raw material for natural selection to act upon.

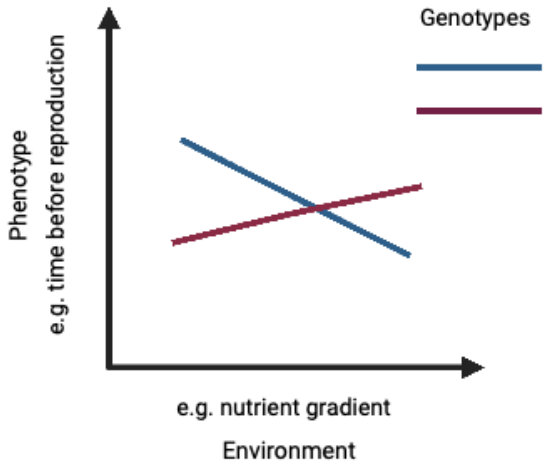


Figure 1.1. Figure showing a reaction norm with a hypothetical GXE interaction. Two genotypes are shown in colours, blue and red.

Parasites are excellent systems to explore phenotypic plasticity and GxE because evolution can be investigated in real-time (parasites generally have shorter generations than their hosts), and we can make experimental setups to create designs (like common garden experiments) to disentangle the contributions of genetic and environmental variation to phenotypes. Furthermore, thanks to their applied relevance, many sophisticated tools have been developed to uncover molecular and genetic mechanisms underpinning parasite traits, that can be used to understand APP at different levels of biological organisation. One of the most used parasites for this purpose are *Plasmodium spp.,* the causative agents of malaria. For example, *Plasmodium* parasites follow the same general rules as multicellular organisms for trading off resources between allocation to replication and reproduction (Schneider, et al., 2018) and between investment in male and female transmission forms (Reece et al., 2008). Building on this foundation, recent work has investigated whether APP allows parasites to cope with constraints imposed by (or exploit opportunities created

by) the daily rhythms of hosts and vectors. My thesis takes these concepts further, to provide insight into *Plasmodium* strategies across the life cycle.

2. *Plasmodium* spp. and life cycle

Plasmodium parasites are important to study because they cause malaria, but additionally, they have proved a great model system for eco-evolutionary parasitology (Paul et al., 2003). *Plasmodium* parasites' historical relationship with human evolution has resulted in abundant knowledge of these parasites across a multitude of hosts. The use of *Plasmodium* spp. in rodent hosts is a very tractable system for exploring host-parasite interactions *in vivo* and in real-time (Kirkman and Deitsch, 2020;Srivastava et al., 2016; Philip, 2022). Since their discovery in Central Africa as parasites of Thicket rats, e.g. *Grammomys surdaster*,(Killick-Kendrick & Peters, 1978) similarities between *Plasmodium* spp. in rodent and human studies have allowed researchers to identify host and parasite factors as well as their interactions that underpin disease phenotypes in human malaria infections (Huang et al., 2018; Zuzarte-Luis et al., 2014).

2.1 Rodent malaria models

The rodent malaria species group is composed of four major species *P. berghei*, *P yoelii*, *P. vinckei* and *P. chabaudi*. These parasites possess a conserved life cycle (Figure 1.2) with minor differences highlighted in several opinion and review articles (Stephens, et al., 2012; De Niz and Heussler, 2018; Simwela and Waters, 2022). Some of these differences include preference for red blood cells (RBC) of different ages, the duration of asexual replication cycles, and patterns of withdrawal from the circulation (sequestration) at certain points in development, among many others. The rodent specie *P. chabaudi* is known to recapitulate several similarities with *P. falciparum* infection dynamics and both possess phenotypic synchrony during asexual replication (Stephens, et al. 2012). However, aspects of research that are common across rodent and human malaria parasites is that the ecology of the liver phase, sequestration, and the mosquito phase are poorly understood compared to the blood phase (Vaughan and Kappe, 2017;Hentzschel and Frischknecht, 2022).

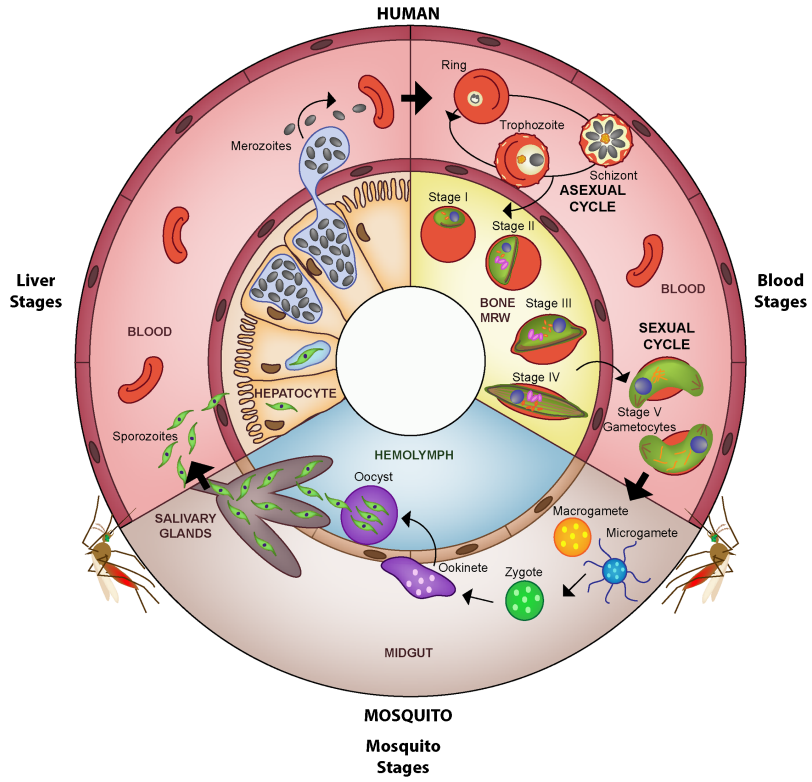


Figure 1.2. Lifecycle of *Plasmodium falciparum* illustrating the vertebrate and vector phases of the life cycle and the different developmental stages that occur within each phase. Figure credit to (Nilsson, et al., 2015).

All *Plasmodium* spp. follow a similar overall life cycle with some deviations (Figure1.2). After inoculation of sporozoites by a blood-feeding mosquito, *Plasmodium* parasites migrate through the vertebrate host's dermis until they encounter the bloodstream, arriving at the liver via passive transport (Douglas et al., 2015). Sporozoites enter hepatocytes, through receptor recognition. Here rodent parasites use different receptors (De Niz and Heussler, 2018), for example, CD81 is used by both *P. yoelii* and *P. berghei* but SRBRII can be used by *P. yoelii*, but in other species receptors have not been characterized (i.e., *P. chabaudi*). After transmigrating through several hepatocytes (speculated to be driven by intraspecific competition(Arias et al., 2022), sporozoites settle into their resident hepatocyte. After sporozoites go through a des-differentiation phase, they enter an extensive replication phase (Heussler et al., 2017). The liver stage duration varies slightly between rodent species (Stephens, et al., 2012), taking ~ 2 days. Every infected hepatocyte produces thousands of merozoites (minimum of ~10,000) that are released into the blood to invade RBC and initiate the symptomatic blood phase.

In the blood, parasites complete successive rounds of asexual replication, with each mature parasite producing ~ 4 to 12 progeny, depending on the species (Mideo et al., 2011). The length of this intraerythrocytic developmental cycle (IDC) also varies between species but generally lasts multiples of ~24 hours (Mideo et al., 2013). From each round of asexual replication, a few (0.01–1% (Carter et al., 2013)) parasites are destined to become transmission stages (i.e., gametocytes) which differentiate into gametes when taken up in a mosquitoes blood meal, subsequently male and female gametes mate and fuse, forming a zygote. Zygotes differentiate into ookinetes, a motile form that migrates through the basal layer in the midgut, forming an oocyst (Hentzschel and Frischknecht, 2022). After many mitotic divisions within oocysts, sporozoites are released and migrate to the mosquitoes' salivary glands to initiate new infections when the mosquito blood-feeds again.

3. *Plasmodium* life cycle in a co-evolutionary context

During their complex life cycle, *Plasmodium* are confronted with different host taxa (a vertebrate host and an insect vector), different tissues within each host (skin, liver, blood in the vertebrate; the gut, haemocoel and salivary glands in the insect), and these environments change as infections develop over days and weeks as well as during daily, 24 hour cycles. My thesis asks how parasites have evolved to cope with the challenges of life in such variable environments, and how they exploit the opportunities that their lifestyle brings. My research has focused on uncovering the basic ecology of neglected aspects of the life cycle, as well as tackling aspects that are well researched but remain poorly understood. This includes the liver phase and sequestration, and interactions with innate immune defences. In this section, I briefly describe mammalian host defences and immune-mediated challenges in the liver and the blood, before incorporating the role of daily rhythms in immune responses and feeding-fasting.

3.1 Immunity during the liver stage

The liver phase was initially thought of as a “silent” phase (Zuzarte-Luis and Mota, 2020), yet there is increasing recognition that immune responses are directed against invading sporozoites and infected hepatocytes (Liehl and Mota, 2012). Much of the knowledge of immune responses to the liver (exo-erythrocytic) stages comes from efforts to create a sterilizing vaccine and can be summarised as follows. The humoral response (i.e. antibody-mediated mechanisms) inhibit sporozoites through different mechanisms, including reducing their motility and/or ability to invade hepatocytes (Abuga et al., 2021). The cellular response, mainly driven by CD8 T cytolytic activity, mediates clearance of infected hepatocytes, although CD4 T cells can enhance, and to a lesser extent engage in, the cytolytic activity (Abuga et al., 2021). T cells can directly kill infected hepatocytes or indirectly act through cytokines (e.g. IFNγ) that promote nitric oxide (NO) which directly kills parasites within hepatocytes. Host-parasite interactions in the liver phase do vary across *Plasmodium spp;* for example, CD8 T cells respond to *P. berghei* mainly with IFNγ and TNFα, whereas hosts infected with *P. yoelii* rely more on IFNγ and perforins (Urban et al., 2017). These identified immune factors (recognition receptors and effectors) have daily rhythms, and when coupled with parasite specificities, we might expect species-specific effects during the liver stage.

3.2 Immune defences during the blood phase

Unlike the liver phase, the blood (erythrocytic) phase has been historically associated with cyclical fevers and strong inflammatory responses (Götz et al., 2017). Despite more intense study in rodent models and humans (often in culture conditions) than the liver phase, the complex nature of the mammalian immune system has resulted in conflicting reports of how innate immunity acts against *Plasmodium* parasites in the blood (Götz et al., 2017). Immune responses can be separated into two main branches innate and adaptive (Abbas et al., 2015). Innate immunity compromises the host's first line of defence and is mounted during the first encounter with a parasite, whereas adaptive immunity requires activation and occurs after approx. 7 days following infection (Abbas et al., 2015). I focus on innate immune mechanisms because literature has found conflicting mechanisms of action and impacts on parasites, and

because these mechanisms are expected to contribute mainly during the early phase of infection.

Plasmodium parasites produce several molecules that the host quickly recognizes, including GPI, hemozoin, dsRNA, among many others (Gazzinelli et al., 2014). These molecules are recognized by host pattern recognition receptors (PRR), including toll-like receptors (TLRs)(Gowda, 2007; Gowda & Wu, 2018) and several cellular innate immune factors are activated (Urban et al., 2005). The adaptive immune response mainly involves specific antibodies involving specific cellular types like T and B cells. Humoral responses (e.g. antibodies) can block merozoites by inhibiting RBC invasion or enhancing phagocytosis/complement-mediated destruction (Rogers et al., 2021). To control the replication of *Plasmodium,* an early Th1 response characterized by IFNγ production and a timely shift from Th1 after the first peak of parasitaemia to the production of specific antibodies is required (Stephens, et al., 2012). Multiple players are involved in coordinating such immune responses. Immune cells play a variety of roles, including:

1) Macrophages are key during the acute phase (Chua et al., 2013), playing a direct role against parasites through phagocytosis in the absence of opsonization or antibodies (Götz et al., 2017). Yet, macrophages can become dysfunctional upon encountering *Plasmodium* (Chua et al., 2013), meaning *Plasmodium* parasites interfere with this cell activity. However, depleting this cell type reveals it is dispensable because it does not alter early infection dynamics.
2) Dendritic cells act as intermediaries of innate and adaptive immune responses. Upon activation, dendritic cells mediate lymphocyte activity through antigen presentation (Yap et al., 2019). Besides producing cytokines and activating cells, their main role is as antigen-presenting cells and conflicting findings about causing dysfunctional phenotype with impaired maturation have been found (Stevenson and Riley, 2004).
3) Natural killer (NK/NKt) cells' main role is through interferon-gamma (IFNγ) production to curb parasite replication in multiple ways (Götz et al., 2017). Removing these cells is not lethal to the host. Yet, removing pro-inflammatory cytokine IFNγ can be fatal, causing delayed clearance with enhanced parasitaemia (Inoue et al., 2013).

4) γδ T cells are a small proportion of circulating T cells. Their role in anti-*Plasmodium* activity is unresolved but is suggested to be indirect, acting through cytokines like IFNγ (Inoue et al., 2013).
5) Among T cells, CD4 T cells control the peak of parasitaemia by inducing high levels of IFNγ during the acute phase (Wykes et al., 2017). Whereas CD8 T cells' role during the blood stage is more controversial, it is likely related to enhancing parasite clearance in mouse models (Wykes et al., 2017).
6) B cell's role in humoral responses after the initial antibody response is dysregulated under *Plasmodium* infection but the mechanisms are not well understood (Rogers et al., 2021). Such dysregulation could potentially explain why long-term immunity is so hard to acquire. In mice, B cell depletion causes infections that are unable to be cleared and alter the dynamics of the primary acute phase (von der Weid et al., 1996).

Despite the established wisdom, many questions remain unresolved, like what causes the dysfunctional phenotype of some immune cells, and what parasite-specific mechanisms interfere with host immune responses? Some literature reviews have pointed out that unresolved questions and discrepancies found in the field might be due to methodological approaches, not biological factors, for example, discrepancies between *in vitro* and *in vivo* studies of the same immune mechanism. However, it is not known if other biologically overlooked traits might be driving variation across studies, like circadian rhythms in immune responses. Almost all components of the mammalian immune system are under tight circadian control (Curtis et al., 2014; Hunter et al., 2022; Wang et al., 2022). Most classes of immune cells possess intrinsic clock machinery that can be entrained by rhythmic signals (Palomino-Segura & Hidalgo, 2021). To what extent daily rhythms in innate immune components are robust to the insults of acute infection, or even helpful in such circumstances, are unknown.

3.3 Rhythmic hosts

Biological rhythms, specifically circadian rhythms, have evolved to anticipate the ~24 hourly rotation of the Earth (Vaze and Sharma, 2013). Most of the daily rhythms exhibited by mammalian hosts and insect vectors are driven by circadian clocks. Circadian clocks require a ‘‘Zeitgeber’’ or time cue to ‘‘entrain’’, have a built-in ability

to anticipate daily environmental changes and are temperature compensated, enabling them to tick at the correct pace across a biologically relevant temperature gradient (Saini et al., 2019). These features persist due to clock-control even in the absence of environmental rhythms and ‘‘free run’’ with a duration (“period”) of approximately 24 hours (Figure 1.3). The features ensure that clock oscillators balance being robust to perturbation while being flexible enough to keep up with for example changing photoperiod across seasons.

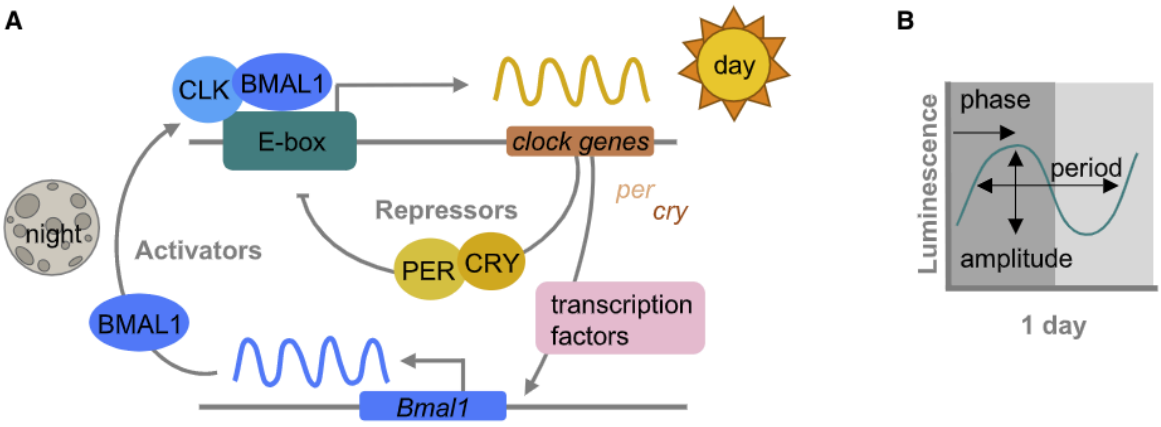


Figure 1.3. A) Main molecular components of the TTFL, transcription-translation feedback loop, of the mammalian cellular clock components. The complete cycle of transcription, translation and degradation takes around ~24 hours. The proteins CLOCK (CLK), BMAL1, PERIOD1and PERIOD2 (PER), and CRYPTOCHROME1 and CRYPTOCHROME2 (CRY). B) Characteristics of rhythmic oscillation of gene expression illustrated by a luminescence readout, ‘‘period’’ (~24 hours, one cycle), ‘‘phase’’ (time relative to external reference), and ‘‘amplitude’’ level of expression (the magnitude from peak to trough). Figure credit (Prior et al., 2020).

At a molecular level, the canonical circadian clock consists of self-sustained oscillations in molecular components called transcriptional-translational feedback loops (TTFL). The transcription, translation and degradation of these molecular components takes around ~ 24 hours. In mammals, the core molecular components of the TTFL are BMAL1, CLOCK, PER1/2 and CRY, and different players carry out similar roles in other taxa (Saini et al., 2019). Within a multicellular host, almost every cell possesses its own clock and so their schedule (“phase”) must be synchronized (Albrecht, 2012); indeed, the dysregulation of clocks in different organs caused by jet-leg and shift work patterns has unpleasant manifestations, for example, phenotypic manifestations that are experienced when there is a misalignment between physiology and activity rhythms. Moreover, synchronisation allows for competing or incompatible functions (e.g. DNA replication and metabolism) to be temporally compartmentalized

from each other (referred to as intrinsic adaptive value)(Vaze and Sharma, 2013). In mammals, temporal coordination involves the suprachiasmatic nucleus (SCN) being entrained by light and communicating timing information to peripheral clocks in the rest of the body through several signals (Mohawk et al., 2012; Zhang et al., 2020). Other time cues, such as feeding-fasting rhythms, can also entrain clocks in some organs and provide timing information for the SCN to integrate (Damiola et al., 2000).

Given the pervasive influence of circadian clocks (e.g. ~40% protein-coding genes oscillate in at least one organ, (Zhang et al., 2014)), *Plasmodium* parasites are confronted with daily rhythms in many aspects of host physiology and behaviour, in addition to immune responses. This includes host activity patterns and feeding-fasting schedules, as well as their consequences for metabolism and body temperature. Whilst rhythms in immune factors can create predictable daily windows of dangers to parasites, feeding rhythms generate a window during which essential nutrients can be acquired and a window when resource limitation may be a risk (Prior et al., 2020). Furthermore, rhythms in metabolism affect how quickly some antimalarials are metabolised into their active form and their half-lives, adding a further dimension to daily dangers. Understanding to what extent parasites must simply withstand the effect of these rhythms versus to what extent they can alter their own daily rhythms to cope with challenges and maximise opportunities is a growing area of research (Owolabi et al., 2021).

4. *Plasmodium* strategies for within-host survival

In this section, I outline how viewing *Plasmodium* parasites through an eco-evolutionary lens can uncover the strategies they use to maximise within-host survival as well as reveal constraints on parasite strategies that can be exploited to control them. I start by summarising general concepts before exploring strategies relevant to evading immune defences, using the liver as a niche, and coping with host circadian rhythms.

4.1 General concepts

The different environments and stages that *Plasmodium* parasites transition through generate different ecological constraints and opportunities. For example, different compartments within the host vary in oxygen availability and how much oxidative stress they impose on parasites, and parasites at different developmental stages vary in how much oxygen they require and their vulnerability to oxidative damage (Briolant et al., 2007). Resolving a trade-off between the benefits of oxygen requirements against the costs of oxidative damage may manifest as favouring parasites that reside in certain environments but only at particularly robust developmental stages.

The evolution of traits expressed by one parasite stage is not necessarily independent of how this trait affects other parasite stages, for example, during the blood stage, parasites can scavenge certain lipids, but they must synthesize during the mosquito/liver stage (Shunmugam et al., 2022). Yet, there are common basic needs that parasites need to fulfil regardless of what developmental stage or life cycle phase they are in. Some strategies for fundamental physiology are conserved across species, whereas others have diverged over evolutionary history. For example, *Plasmodium* spp. need to scavenge essential ammino-acids from breaking down haemoglobin and from the host's digestion of food because the phylogenetic group lacks the pathways/enzymes to synthesize ammino-acids such as arginine, isoleucine, methionine (Krishnan and Soldati-Favre, 2021). Yet, the amino acid contents of haemoglobin vary between host species (and ages of RBCs), opening up the possibility for *Plasmodium* spp. to vary in the details of amino-acid usage and acquisition. In terms of haemoglobin breakdown, another selective driver is suggested to be the need for osmotic stability (Lew et al., 2003) which also creates space within the RBC for the growing parasite and releases some amino acids as a by-product, obscuring the role of selection purely for nutrient acquisition.

To cope with different environments with the host and the changes that occur within each environment as infections progress, parasites use APP to adopt flexible phenotypes. For example, anaemia and immune responses develop over the days and weeks of the blood phase of infection, drug treatment may occur, and incoming parasite genotypes can cause within-host competition. Parasites make the best out of these situations by responding to changes in environmental factors by adjusting their investment in asexual versus sexual stages, and between male and female sexual

stages in the manners that maintain fitness (reviewed in Schneider and Reece, 2021). Whilst APP has received the most attention in the context of parasite reproductive strategies, asexually replicating stages in the IDC also appear to alter their traits. For example, being able to change the timing (phase) of replication with respect to host daily rhythms by shortening the duration of the IDC (O'Donnell et al., 2022), altering RBC age preference and the number of progeny per mature schizont in response to the age distribution of RBC resources (Birget et al., 2017). However, whilst life history theory has provided well-verified explanations for why plasticity in parasite reproductive strategies is adaptive (Schneider et al., 2018), the costs/benefits of plastic alteration of IDC stage traits are not well understood.

4.2 Strategies for within-host survival across parasite stages

Focusing on the strategies *Plasmodium* parasites have evolved to survive and proliferate in the host, antigenic variation is one of their best-known tactics. Var genes are required for cytoadhesion which underpins the sequestration required to evade splenic clearance, yet the expression of proteins trafficked to the RBC surface exposes antigens to the host (Berendt et al., 1994; Roberts et al., 1993; Sherman et al., 2003). To overcome the host's ability to recognise and mount a specific antibody response to a particular var gene, *Plasmodium* has the ability to switch var expression every IDC, even a small proportion of parasites expressing a different var gene possess new antigenic properties (and also cytoadhesive properties), enabling the parasite to proliferate whilst the host's acquired defences catch up (Rénia and Goh, 2016). Thus, antigenic variation enables parasites to evade both splenic clearance and specific immune responses. Antigenic variation is an example of APP because phenotypic variation is generated within individual genotypes. Several gene families are responsible for this parasite strategy, PIR, RIFIN, STEVOR, and var genes, depending on the *Plasmodium* species (Sherman et al., 2003). In the human parasite, *P. falciparum,* the var genes have been explored in most detail, and are thought to have tightly regulated expression so that only one variant type is expressed and displayed (Duraisingh and Skillman, 2018).

Another plastic strategy adopted during the blood phase is dormancy. This can be adaptive when parasites enter a quiescent, mostly ametabolic stage, arresting IDC progression, to survive periods of stress. Dormancy has been described in response to cytotoxic stress induced by antimalarial drug treatment (mainly described against artemisinin derivates)(Teuscher et al., 2010), in which young IDC (ring) stage parasites can persist for multiple days. Parasites may also enter a form of dormancy when starved of isoleucine which as well being an essential resource, is also used as a time-cue to enable parasites to synchronise their IDC schedule with the host's feeding-fasting rhythm (Babbitt et al., 2012; Prior et al., 2021). Other forms of dormancy include the resistant hypnozoite stage produced by *P. vivax*. Hypnozoites are persistent liver stages that can re-seed a blood infection several years after the initial infection (Markus, 2015). Hypnozoites appear to be a form of insurance, but whether unpredictable or spread-out transmission seasons and/or the risk of blood stage clearance have selected for this strategy is unknown. In contrast, *P. relictum*, an avian malaria, can detect in the onset of a transmission season and upregulate some (unknown) activities to improve infectivity to mosquito vectors (Cornet et al., 2014).

As previously mentioned, parasites adjust investment into sexual stages (conversion rate) when experiencing unfavourable conditions. They prioritise survival by reducing conversion (adopting reproductive restraint) to maximise asexual replication within host conditions become adverse (e.g. when experiencing with-host competition or low doses of antimalarials). But if circumstances become so harsh that in-host survival is unlikely, parasites maximise conversion as a form of terminal investment (Schneider and Reece, 2021). In the case of conversion rate plasticity, parasites are adopting a context-dependent strategy, based on responding to cues informing them of their circumstances. In contrast, rates of antigenic variation and dormancy might not be actively altered in different situations, but instead, these activities are something a set proportion of parasites is programmed to undertake irrespective of circumstances. Nonetheless, plasticity in various traits clearly enables parasites to cope with multiple stressors within the host, including intraspecific competition, immune attack, antimalarial drugs, and RBC limitation. Recent evidence also suggests parasites use APP in the rate of IDC progression to cope with environmental variation caused by daily rhythms of the host.

4.2.1 Rhythmic parasites

Historically *Plasmodium's* rhythmic replication patterns were recognized because of the periodicity in the fevers caused in their mammalian host (Garcia et al., 2001). Later, periodic patterns of fever were discovered to be caused by the rhythmic bursting of parasites when each cohort synchronously replicating asexuals stages completes its IDC. This rhythmic IDC progression occurs in many *Plasmodium* spp., resulting in IDC durations (periods) lasting 24, 48, or 72 hours (Mideo et al., 2013). For each IDC, the parasite can be classified into three main developmental stages inside their resident RBC. First the ring stage: as soon as they enter their main task is to remodel the cell. Second, the trophozoite stage: parasites grow and synthesize DNA for their daughter merozoites using resources (including amino acids and glucose) to complete these tasks. Third, the schizont stage: the differentiation into daughter cells (merozoites) which requires lipids to form each membrane (Prior et al., 2021). Because specific stages require specific resources, transitions can be scheduled to match when these requirements can be met. Thus parasites actively coordinate the IDC schedule with the timing of host circadian rhythms to transition between stages at a specific time of day (Figure 1.4). Two independent research groups found a consistent link between feeding-fasting rhythms and the rhythmic IDC progression (Hirako et al., 2018; O'Donnell et al., 2020; Prior et al., 2018). They demonstrated that parasites align their IDC to the feeding-fasting rhythm irrespective of the timing of host rhythms driven by photoperiod, and whether or not the host has a functional TTFL.

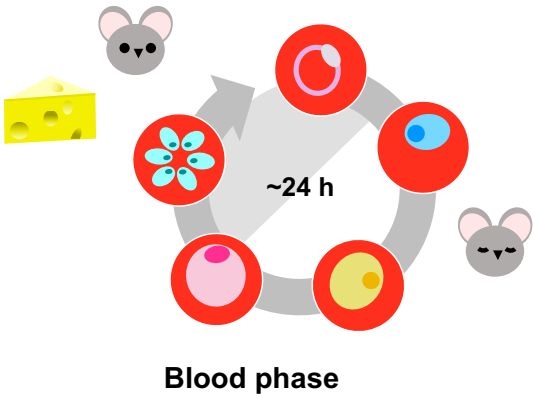


Figure 1.4. The asexual replication cycle and its timing are related to its host rhythms: photoshedule (dark – grey shade) and food (cheese) and activity (awake/rest).

Precisely why *Plasmodium* aligns the timing of transitions between stages in the IDC to host rhythms is mysterious. The emerging consensus from *P. chabaudi* is that the IDC schedule is timed to maximise both the exploitation of host resources and infectivity to the mosquito vector (O'Donnell et al., 2011; O'Donnell et al., 2013; Schneider, et al., 2018). Specifically, the IDC appears timed to ensure that parasites transition to the developmental stages that need the most nutrients from the host's food during the feeding window (Prior et al., 2021). And conveniently, this schedule may also result in sexual stages reaching maturity at night, when mosquito vectors forage for blood. Whilst the specific benefits are yet to be unequivocally demonstrated, the costs of an IDC schedule that is misaligned to host rhythms include a reduction in sexual stage density, and loss of replicative capacity of asexuals (O'Donnell et al., 2011). Misaligned parasites rapidly reschedule by shortening the IDC (by approx. 2 hours per cycle), yet this faster replication rate does not result in higher densities over successive IDC than exhibited by aligned parasites (O'Donnell et al., 2022). This suggests that a shorter IDC comes at a cost of fewer merozoites per schizont, or RBC invasion at the "wrong time of day" is less successful. But given that parasites pay these costs to reschedule the IDC, they must be offset by significant benefits of being on time.

How parasites tell the time and synchronise development across each IDC cohort is also mysterious. Some parasites possess a circadian oscillator. For example *Trypanosoma brucei*, the causative agent of sleeping sickness, possesses an oscillator entrained by temperature that controls rhythmicity in the expression of many metabolic genes (Rijo-Ferreira et al., 2017). The fungal plant pathogen *Botrytis cinerea* also possesses a circadian oscillator, with orthology to Neurospora sp.'s

oscillator, that mediates the amount of damage caused to its plant host (Larrondo and Canessa, 2019). *Plasmodium* exhibits some characteristics consistent with circadian oscillators too (Rijo-Ferreira et al., 2020; Smith et al., 2020), but temperature compensation is yet to be tested. Furthermore, interpreting the maintenance of rhythmicity within the IDC in the absence of host rhythms is complicated; it is consistent with parasites' free-running (as concluded in Rijo-Ferreira et al., 2020), but knowledge of how much natural variation exists in the IDC duration (period) is required to estimate how long rhythmicity will persist without an oscillator. Put another way, do IDC rhythms dampen in hosts without a TTFL because they are free running, or because the IDC is constrained to be close to 24 hours in the absence of time cues and so, natural variation in IDC duration takes many cycles to reduce synchrony.

Timekeeping does not always require a sophisticated circadian oscillator – a just-in-time, reactionary (i.e. APP) strategy is sufficient for other species, albeit without the benefits of anticipation or temperature compensation. However, the consensus is that *Plasmodium* parasites are actively able to modulate their IDC schedule; the gene, serpentine receptor 10 controls IDC duration (Subudhi et al., 2020), and patterns of rescheduling cannot be explained by a direct, enforcing, impact of host rhythms (i.e. mistimed IDC stages do not have a substantially higher mortality risk). Indeed, for *P. chabaudi*, the concentration of isoleucine in the blood is rhythmic because it is food derived, being essential for both host and parasite, and is sufficient to act as a time of day cue for the IDC schedule (Prior et al., 2021). Time cues may also involve liver-derived glucose oscillations induced by TNFα in response to malaria inflammation (Hirako et al., 2018). Testing whether isoleucine (and other time cues) acts as a Zeitgeber or is simply a signal for a just-in-time strategy is challenging.

Whilst host feeding-fasting rhythms are clearly key to the IDC schedule, how these rhythms affect parasites developing in the liver – the main organ involved in metabolic rhythms (Reinke and Asher, 2016) – is unknown. Also, in addition to generating rhythmicity in nutrient availability, feeding-fasting rhythms are in phase with rhythms in body temperature, blood oxygen tension, and redox, which may all mediate the costs and benefits of particular IDC schedules. Finally, whether rhythmicity in immune responses impacts on either liver or blood stage parasites is poorly understood. It

might be beneficial for the host to override immune cell clocks when responding to the severity of malaria infections, but recovery could be aided by reinstating timing to help restore homeostasis. These topics motivate the questions I have addressed in my thesis.

5. General aim and thesis structure

This thesis explores the role of within-host ecology in shaping phenotypically plastic parasite strategies. Specifically, I use rodent *Plasmodium* models to investigate the role of host biological rhythms and immune defences on the ecology of three poorly understood traits: the transition between liver and blood phase, sequestration, and parasite multiplication rate (PMR) during the early acute phase of infection.

My thesis is structured into five chapters: the introduction and general discussion, and three data chapters. In this first chapter, I have introduced the topics covered throughout the other chapters, demonstrating how an evolutionary ecology framework can be applied to interrogate parasite-host interactions and identified the knowledge gaps I address in chapters 2-4, before a general discussion in chapter 5. The questions I ask in chapters 2-4 are:

> Chapter 2: Do host rhythms influence parasites' transition from the liver into the blood phase, and do rhythms in the liver phase have fitness consequences for parasites?
> Chapter 3: Do host rhythms influence sequestration rates and have implications for parasite fitness?
> Chapter 4: Do host innate immune responses influence parasites' ability to replicate during the establishment of the blood phase of infection?

The specific motivations behind chapters 2-4 is as follows:

5.1 Why investigate rhythms during the liver stage?

In chapter 2, I explore parasites' transition from the liver stage into the blood. The first replicative phase inside the mammalian host takes place in the liver, this is an elusive

stage because of the intrinsic difficulties in studying it. The liver is the main driver of metabolic rhythms within a mammalian host and feeding-fasting rhythms are key for the parasite's IDC to be rhythmic and synchronised to host's (and also the vector's) daily rhythms (O'Donnell 2020, 2021; Prior 2018, Hirako 2018). Thus, liver phase parasites are strongly subjected to the effects of feeding-fasting rhythms on host cells and have abundant information about the timing (phase) of host feeding that they could use to initiate the blood phase "on time". Therefore, I carried out a series of experiments to explore whether feeding-fasting and/or light-dark driven rhythms play a role in the timing of when the blood phase is initiated, and whether the pattern of blood phase establishment has implications for fitness. Parasites in the liver cannot be directly observed for prolonged periods and studying a transition between life cycle phases requires an *in vivo* approach. This introduces caveats to studying liver phase development which I address in the general discussion. However, my approach and results demonstrate that studying the ecology of the liver phase is tractable and opens up new questions to address.

5.2 Why study the effect of within-host rhythms on parasite sequestration?

In chapter 3, I assess the role of feeding-fasting and/or light-dark driven rhythms in the timing of a key parasite immune evasion strategy – withdrawal from the circulation to adhere to endothelial cells in tissues (called "sequestration"). Several studies have identified that variation in within-host conditions relates to variation in parasite sequestration activities, yet these observations' ecological and evolutionary relevance remains obscure. For example, to our knowledge, no studies have formally quantified the costs and benefits of sequestration, nor whether these consequences are context-dependent. For example, sequestration might remove parasites from a nutrient-rich environment, but the costs of avoiding clearance by the spleen outweigh the benefits of readily available nutrients. Alternatively, sequestration might allow parasites to evade the dangers of the spleen whilst also evading the higher risk of oxidative damage in the blood when the host is metabolising (during the active phase). In all of these scenarios - the nutrient content of blood, redox state, and splenic activity - are all governed by daily rhythms. Similarly, to studying liver-phase parasites, sequestered parasites cannot be observed in real-time which introduces caveats to studying

sequestration and its consequences which I address in the general discussion. However, by testing whether host rhythms affect sequestration activities and consequences for replication, my experiment demonstrates that the ecology of the sequestration is tractable and opens up new questions to address.

5.3 Why quantify the effect of innate immune mechanisms across spp.

In chapter 4, I explore how the host's innate immune responses impact parasite multiplication rate. My two previous projects were in part inspired by the potential for innate immune responses to impact on liver-phase development and underpin the fitness benefits of sequestration, and for host circadian rhythms to mediate these phenomena. Thus, these projects assume that innate immunity plays an active role against *Plasmodium* parasites in the early stages of infection. While the immune system is the key player against invading parasites from the host's point of view, most research has focused on identifying which components of the host immune response relate to infection outcome or disease severity. However, generalizing the overall effect innate immune interventions on parasite replication during the acute phase has not been properly addressed nor quantified. Therefore, I applied a meta-analytic approach to quantify how much innate immune interventions impact rodent *Plasmodium* spp. acute infection dynamics. Furthermore, I also applied meta-regression to identify sources of variation during innate immune intervention across rodent malaria species to help resolve conflicting observations in the literature; for example, testing whether certain immune components have different impacts on different *Plasmodium* species, and whether different methodologies are associated with different outcomes. Meta-analysis approaches are commonly used to provide a quantitative synthesis of a body of literature in evolutionary ecology because many studies have large sample sizes and report standardised metrics. Unfortunately, a high proportion of the studies I screened could not contribute to my analysis, highlighting that to benefit from the utility and power of meta-analysis approaches, parasitology/immunology studies require better study designs and more rigorous reporting.

In chapter 5, I develop a general discussion, including an outlook and the relevance of my research and model system for malaria control. I also discuss the limitations of my approaches, including the compromises and assumptions with my approaches required, and I conclude with future directions derived from my projects.

Chapter 2. *Plasmodium* parasites transition from replication in the liver to the blood-stage independently of host daily rhythms

Abstract

To establish an infection in a mammalian host, *Plasmodium* spp. must replicate extensively in the liver before invading the bloodstream. Liver cell biology is governed by circadian rhythms due to its key role in food digestion and metabolism, generating daily rhythms in the nutrients available to blood stage parasites. Consequently, *P. chabaudi* aligns its blood-stage replication with the timing of host feeding-fasting rhythms to maximise both exploitation of within-host resources and infectivity to mosquito vectors. We test whether *P. chabaudi's* activities in the liver are also scheduled to align with host feeding-fasting rhythms, predicting that such a strategy benefits parasites fitness by enabling the blood stage to begin “on time”. We expected parasites to egress from the liver and colonise the blood in a synchronous pulse is at the end of the feeding window because that is the best time-of-day to begin blood-stage replication. However, both parasite genotypes tested accumulate in the blood in a manner insensitive to rhythms driven by either the host’s feeding-fasting schedule or its canonical circadian clock. Instead, following egress from the liver, blood stage parasites accumulate over many hours following patterns that differ between genotypes. We then reveal that transitioning to the blood stage in a manner independent of host rhythms is a not a disadvantageous constraint imposed by host physiology. Specifically, blood-stage replication is not affected by experimentally simulating egress at different times of day, in either a single pulse or over a prolonged time window. Thus, the rhythmicity in blood stage *Plasmodium* replication that famously causes regular bouts of fever is rapidly established only once parasites enter the blood. This hitherto unknown plasticity in parasite strategies informs how to perturb the timing of host-parasite-vector interactions for clinical benefit.

Introduction

Malaria (*Plasmodium*) parasites have evolved a suite of strategies to exploit the opportunities of their lifestyle and cope with the challenges it brings. This includes the ability to alter traits and behaviours during infections in response to changes in the environmental conditions they experience inside their hosts. Such adaptive phenotypic plasticity (APP; the ability of organisms to alter aspects of phenotype to maintain fitness in the face of environmental change (Pfennig and West-Eberhard, 2021) has been well documented for *Plasmodium's* strategies for producing sexual transmission-stages. For example, investment into sexual versus asexual stages is adjusted during infections in response to a multitude of factors including in-host competition, anti-malarial drugs, and anaemia, in manners that maximise fitness (Schneider and Reece, 2021). Additional abilities include investing in transmission in response to the seasonal increase in vector population size (Cornet et al., 2014), and altering the ratio of male to female sexual stages in response to their inbreeding rate and how likely males are to be able to locate and fertilise females when mating in the vector's blood meal (Reece et al., 2008). Empirical and theoretical evidence supports that these "reproductive strategies" are encoded in the genomes of *Plasmodium* spp. and have evolved to maximise parasite fitness, suggesting that adaptive strategies are deployed by asexually replicating stages in the blood (i.e. during the erythrocytic phase). Such strategies include entering a form of reversible dormancy in response to drug treatment (Paloque et al., 2016), variation in the number of progeny per mature parasite in response to perturbations of host anaemia and nutrition (Birget et al., 2019; Mancio-Silva et al., 2017), and aligning rhythmicity in asexual replication with the timing of the host's feeding-fasting rhythm (Hirako et al., 2018; O'Donnell et al., 2020; Prior et al., 2018). However, whether *Plasmodium* deploys plastic strategies in other aspects of the life cycle, including during the liver (exo-erythrocytic) phase is unknown. Here, we focus *Plasmodium's* ability to schedule its replication to ask whether parasites can alter the timing of activities in the liver to ensure the transition to the erythrocytic phase begins "on time".

The erythrocytic phase is characterised by the rapid proliferation of asexually replicating parasites in successive cycles that take 24, 48, or 72 hours, depending on

the *Plasmodium spp.* (Garcia et al., 2001; Mideo et al., 2013a). This intraerythrocytic developmental cycle (IDC) begins when merozoites invade red blood cells and ends when each mature schizont bursts to release its merozoite progeny. In most *Plasmodium spp.,* development during the erythrocytic phase is synchronous with transitions between stages occurring at particular times of day. For example, bursting of *P. chabaudi* predominantly occurs at night, when its nocturnal rodent host is actively foraging, because the IDC schedule is driven by daily rhythms associated with feeding and fasting (Hirako et al., 2018; O'Donnell et al., 2021; Prior et al., 2018). The molecular mechanisms underpinning the IDC schedule are unknown but its fitness consequences are significant; aligning the IDC with host feeding-fasting rhythms facilitates exploitation of resources from the host's food that are essential for replication, as well as ensuring sexual stages are at their most infective at night when mosquito vectors forage for blood (Schneider, et al., 2018). Given the fitness benefits of a correctly timed IDC, it should be advantageous to begin the erythrocytic phase "on time" - at the point of the transition between the exo- and erythrocytic phases. Exo-erythrocytic parasites are well placed to schedule their egress into the blood according to host feeding-fasting rhythms for two reasons. First, they reside within the main organ responsible for metabolic rhythms and generating rhythmicity in the availability of nutrients that both exo-erythrocytic and erythrocytic stages require for development; indeed over 15% of coding genes in liver cells are expressed with a daily rhythm (Zhang et al., 2014). Thus, exo-erythrocytic parasites have ready access to cues for the timing of the host's feeding-fasting rhythm. Second, exo-erythrocytic parasites might only be able to complete replication at a certain time of day if they are constrained by rhythmicity in nutrient supply from the host's food. Within an infected hepatocyte, extensive asexual replication occurs over approximately 2-7 days, depending on the *Plasmodium spp.,* yielding between 30,000 to 1,000,000 merozoites per hepatocyte. Thus, energetic demands on exo-erythrocytic parasites are high and they rely on resources from their residing hepatocyte, as well as their own synthesis; for example, biasing host transcription processes to their own benefit (Albuquerque et al., 2009). Therefore, if exo-erythrocytic parasites rely on the host's digestion of food for access to essential resources, they may only be able to complete replication at a certain time of day, conveniently ensuring egress to initiate the IDC also begins at the best time of day.

If egress is initiated in response to time-of-day cues, then removing this information or preventing parasite responses could simultaneously prevent disease symptoms and block transmission. To test whether *Plasmodium* begins the erythrocytic phase with the IDC correctly aligned to the host's feeding-fasting rhythm, we carried out a series of experiments using *P. chabaudi.* This rodent malaria model allows parasites to be exposed to the complexity of all daily rhythms and physiological processes that operate within a mammalian host, maximising ecological relevance. At the end of the exo-erythrocytic phase, liver-derived merozoites egress passively in hepatocyte derived vesicles (Douglas et al., 2015), called merosomes (Sturm et al., 2006). Merosome behaviour is not well understood but some *Plasmodium spp.* may travel to organs, such as the lungs, via the bloodstream (Baer et al., 2007), where egress occurs and the first IDC begins. Most studies report that egress occurs over a window of ~10 hours (Baer et al., 2007), but egress is very challenging to quantify (Vaughan and Kappe, 2017). In addition, most studies use parasites such as *P. berghei* that does not align its IDC to any host rhythms making it an inappropriate model for studies of parasite timing (O'Donnell and Reece, 2021). In contrast, *P. chabaudi* should benefit from establishing the IDC in alignment to the host's feeding-fasting rhythm and could achieve this by egressing during a narrow window at a certain time-of-day. Egress could be scheduled in this manner via two mechanisms. The exo-erythrocytic phase has a fixed developmental duration that has evolved to marry the average time-of-day mosquitoes bite to initiate new infections and the optimal IDC schedule. Alternatively, egress may be timed *via* a plastic response to time-of-day cues, manifesting as a flexible duration for the exoerythrocytic phase. By manipulating the time-of-day infections were initiated with respect to feeding-fasting rhythms and host canonical circadian clocks, we tested whether the duration of the exoerythrocytic phase is constrained by a fixed development period and/or plastically adjusted to align with host rhythms. Furthermore, explaining when, why, and how plastic strategies affect parasite fitness requires testing how costs and limits govern their phenotypic traits, yet these aspects are rarely examined (Birget et al., 2019). Thus, we experimentally simulated different egress patterns to test whether the erythrocytic phase is established in an adaptive (i.e. fitness enhancing) manner.

Our experiments reveal that parasites initiate the erythrocytic phase in a genotype-specific manner, but the overall pattern is arrhythmic. Specifically, the timing, pattern,

and rate of accumulation of parasites invading the blood are insensitive to both the feeding-fasting rhythms and canonical (transcription-translation-feedback loop, TTFL) clocks of hosts. Furthermore, neither the timing nor degree of synchronicity of experimentally simulated egress affect the multiplication rate of the first few IDCs, suggesting arrhythmic accumulation in the blood following egress is selectively neutral. Our findings are unexpected but can be explained by recent discoveries of plasticity in the IDC schedule. *P. chabaudi* for example, is capable of rescheduling its IDC by speeding development up by approx. 2 hours per IDC, potentially via repression of the gene Serpentine Receptor 10, allowing it to recover from a 12 hour misalignment to host rhythms within 6 IDC (O'Donnell et al., 2021; Subudhi, et al., 2020). In view of the data presented here, we propose this plasticity in the IDC schedule has evolved as a strategy to rapidly align the IDC to host rhythms following arrhythmic egress from the liver. Because blood-stage replication is responsible for the severity of disease and fuels the production of transmission stages, a better understanding of its ecological context may offer novel interventions to alleviate symptoms and reduce transmission by disrupting parasite timing.

Methods

Parasites, hosts and vectors

All experimental infections consisted of *Plasmodium chabaudi* genotypes AS and AJ and exo-erythrocytic infections were established using *Plasmodium berghei* ANKA in the final, fitness consequences, experiment. Hosts were either wild type C57BL/6 or Per1/2-null mice (non-functional proteins Period 1 & 2, backcrossed onto a C57BL/6 background for over 10 generations), as described in (O'Donnell et al., 2020). Per1/2-null mice are arrhythmic when housed in constant conditions such as constant darkness (Zheng et al., 2001). All mice were mixed sexes, 8–10 weeks old, housed at 21°C and were fed standard RM3 pelleted diet (801700, SDS, UK) with unrestricted access to drinking water supplemented with 0.05% para-aminobenzoic acid (Jacobs, 1964). All mice were acclimated to the light-dark schedules and feeding-fasting regimes for their treatment groups for at least two weeks before their infections with either *P. chabaudi* or *P. berghei*. All procedures were carried out complying with the

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

Mosquitoes were *Anopheles stephensi,* infected and maintained following (Spence et al., 2012), and used to generate the exoerythrocytic phases in all mice. Female mosquitoes between 3 to 5 days post emergence in a single large cage were allowed to feed on groups of mice that had been inoculated with $1x10^5$ RBC parasitised with either *P. chabaudi* AS or AJ or *P. berghei*. For the *P. chabaudi* infections, transmission to mosquitoes were carried out on day 14 post-infection (PI) using mice for which gametocytes were observed on thin blood smears on day 13 PI (AS = 16, AJ = 20). For *P. berghei*, mice were pre-treated with 125mg/kg phenylhydrazine to stimulate gametocytogenesis, which was verified on day 5 PI and transmission to mosquitoes occurred on day 6 PI. Following these feeds mosquitoes remained in large cages, with *P. chabaudi* infected mosquitoes housed at 26°C and *P. berghei* infected mosquitoes at 21°C, 60% relative humidity and fed 8% Fructose and 0.05% PABA.

Before all transmissions to infect experimental mice by mosquito bite, on day 8 (for *P. chabaudi* AS and AJ) and days 13-14 (for *P. berghei*) post mosquito infection, ~10% of mosquitoes per experiment were dissected and stained with mercurochrome 0.05% to verify the presence of oocysts. Following verification of infection, mosquitoes were randomly allocated to pots (on day 13 for *P. chabaudi* and day 20 for *P. berghei*) before being starved overnight prior to infecting experimental mice by placing each anaesthetised mouse on a single pot for 20 mins, following Spence et al. 2012 (Spence et al., 2012). Briefly, during transmission, mice were anaesthetised with a mix of Meditomidine and Ketamine. After 20 min of feeding by mosquitoes, mice were injected with 100 uL of 4% Antipamezole sub-cutaneously to recover. For experiment 1 (AS), oocyst prevalence was 58%, and 20 mosquitoes were allocated randomly from a population of 812 to each of 40 pots, of which 11.6 mosquitoes per pot were infected, on average. For experiment 2 (AJ), oocyst prevalence was 54% and 24 mosquitoes from a population of 1,530 were allocated to each of 62 pots, containing 13.32 infected mosquitoes per pot on average. Sporozoite counts were made from pools of mosquitoes by PCR, targeting 18S rRNA following (Bell et al., 2009), being on average11.97±1.67 sporozoites/fed mosquito for AS and 13.14±3.93 for AJ. No

differences between sporozoite loads were detected among treatments for experiments 1 (AS) and 2 (AJ), with median sporozoite counts for AS of 449.7 (χ^2 (3)= 1.71, p-value = 0.63) and for AJ of 1182.7 (χ^2 (3)=1.68, p=0.64.The mosquitoes used to initiate the infections For *P. berghei* infections in experiment 3*,* oocyst prevalence was 77% and 8 mosquitoes from a population of 304 were allocated to each of 36 pots, containing 6.16 infected mosquitoes per pot on average. Sporozoite presence in mosquito biting each mouse was confirmed was confirmed on day the day of transmission by PCR (Bell et al., 2009).

Role of host rhythms in the transition between the exo- and erythrocytic phases: Experiments 1 and 2

The design illustrated in Figure 2.1 and Table 2.1 tested whether host TTFL driven rhythms and/or host feeding-fasting rhythms affected patterns of parasite accumulation in the blood as a proxy for egress following the exo-erythrocytic phase. We carried out two similar experiments, focused on genotypes AS (experiment 1) and AJ (experiment 2), respectively. For AS, between 33% and 70% of mice in each treatment group became infected (Treatment: $\chi^2(3)=0.49$, p-value = 0.922), reducing the sample sizes given in Table 1 to yield n=4 infections in the Per1/2-RF group (Arrhythmic knockout mice with timed restricted feeding), n=6 in Per1/2-AL group (Arrhythmic knockout mice with ad libitum feeding), n=4 in WT-DL (C57BL6 reverse light schedule) and n=6 WT-LD (C57BL6 standard housing light schedule). Whilst unsuccessful transmissions resulted in an unbalanced design this was unrelated to the treatments so does not compromise our analysis. We based the sampling window on reports in the literature that the exoerythrocytic phase lasts 52-53 hours for *P. chabaudi* (Stephens et al., 2012) and we assume the first IDC takes at least 24 hours. Thus, blood sampling began at 48 HPI, before we expected to detect parasites, and then continued from 60 HPI continued until 80 HPI at intervals of 3-5 hrs. We repeated this experiment with some modifications (Table 2.1) using genotype AJ to increase reproducibility, span the range of virulence observed in *P. chabaudi* (AJ is more virulent than AS) (Grech et al., 2006), and because AJ does not to sequester (Nahrendorf et al., 2021a). All AJ experimental mice became infected, yielding sample sizes as in Table 2.1. Sampling took place every 5 hours starting at 48 HPI, and every

12 after 68 HPI. For both genotypes, each sample consisted of 12 uL of blood taken from the tail vein and we measured RBC (red blood cell densities) densities by haemocytometer, and 10uL were used for DNA extraction to quantify parasite densities. We also monitored the weight of mice to verify those in treatments with time restricted feeding, did not experience caloric restriction (No weight differences, repeated measures ANOVA on TRF treatments, $F(1, 29)= 1.999$, $p= 0.168$). In addition previous research has shown that timed restricted fed mice (TRF) are not limited in the amount of food intake during feeding windows and do not experience weight loss (O'Donnell et al., 2020). Also, because we corroborated host sex did not influence parasite densities ($t= 0.9164$, $p= 0.3596$) we exclude this variable from further analysis.

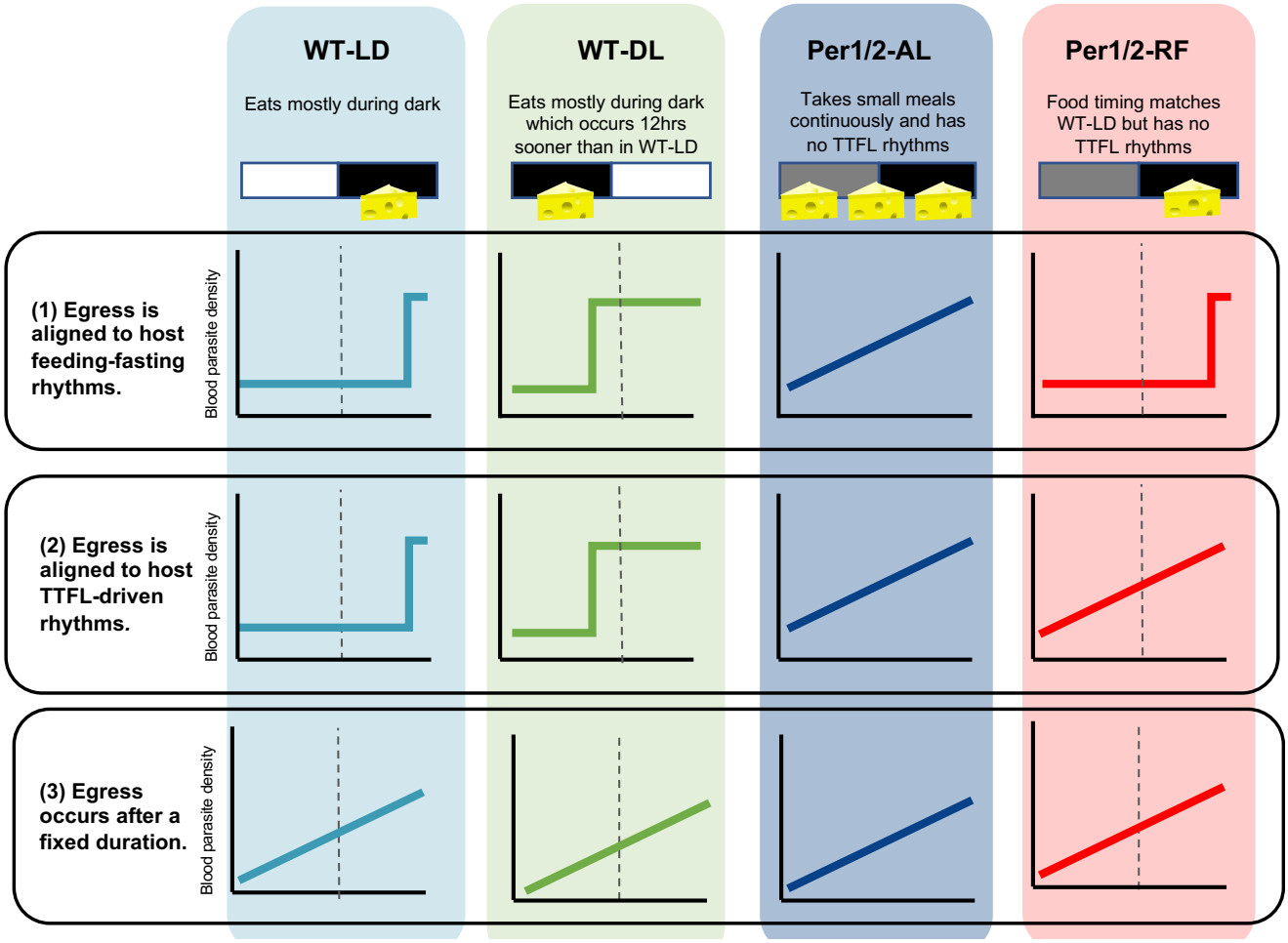


Figure 2.1. Predicted patterns of accumulation in the blood under different egress hypotheses following a liver-phase of ~48 HPI. Each row illustrates the expected dynamics of parasite accumulation in the blood following the liver-phase for each group under a particular scenario, assuming egress is scheduled to begin the IDC "on time". Comparing the timing and shape of densities during accumulation

in the blood allows the hypotheses to be discriminated. Each column represents a treatment group (n=10-15/group) and the key aspects of its feeding-fasting and canonical clock (TTFL) driven rhythms. The x axis represents hours post mosquito bite (HPI) and dotted lines illustrate when transitions in photoschedules and feeding-fasting rhythms occur. If egress is aligned with host feeding-fasting rhythms (hypothesis 1), accumulation is rapid in the 3 groups with feeding-fasting rhythms, occurring towards the end of the feeding window (i.e. 12 hours apart in the WT-DL compared to WT-LD / Per1/2-RF groups) but accumulation is gradual in arrhythmic hosts. If egress is scheduled by host TTFL rhythms (hypothesis 2), its timing is inverted between the WT-LD and WT-DL groups (and must occur at the end of the dark phase for the IDC to begin on time) but in contrast to hypothesis 1, accumulation is arrhythmic in both Per1/2 groups. If egress occurs after a fixed developmental duration (hypothesis 3), either in a short burst or over a prolonged window, parasites will accumulate following the same pattern across all groups. The design for the experiment using genotype AS is illustrated; for AJ, infections in WT mice included a group in which the alignment between feed-fasting and photoschedule driven rhythms is disrupted (WT-LF) and the feeding window in the Per1/2-RF group matched the timing of food availability (GMT) of the WT-LF group.

Table 2.1. Experimental treatment groups. Characteristics of treatment groups and sample sizes for the experiments investigating parasite genotypes AS and AJ. n/a indicates groups that one of the genotypes did not experience. Hosts were either wild type C57BL6 (WT) housed in standard (WT-LD) or reverse (WT-DL) photoschedules, or were Per1/2 null mice, lacking Per 1 and 2 genes with dysfunctional canonical circadian (TTFL) clock and exhibiting arrhythmicity when kept under constant darkness. Hosts were acclimated to their respective photoschedules and feeding-fasting rhythms prior to and during infections. Wild type *ad libitum* fed mice (WT-LD and WT-DL) follow their photoperiod entrained rhythm (i.e. scheduled by the suprachiastismatic nucleus, SCN) to take in most of their food during the dark phase each day, whereas Per1/2 null mice (Per1/2-AL) feed continuously (O'Donnell et al., 2020) unless a feeding-fasting rhythm is imposed by a restricted feeding (Per1/2-RF) treatment. When food is only available to WT mice in their light phase (WT-LF), the timing of their SCN-driven rhythms become misaligned to timing of rhythms scheduled by feeding-fasting rhythms (i.e. peripheral rhythms;(Stokkan et al., 2001)). Note, restricted fed mice were not limited in their amount of food intake during feeding windows and do not experience weight loss (O'Donnell et al., 2020).

Group	AS, n mice	AJ, n mice	Photoschedule (L, Light ; D, dark)	Feeding schedule	TFFL rhythmicity	Feeding activity
WT-LD	10	15	12L:12D, standard AS L: 0800-2000 GMT	*ad libitum*	Entrained	Mostly in dark

			AJ L:0700-1900 GMT			
WT-DL	10	n/a	12D:12L, reverse 2000-0800GMT	*ad libitum*	Entrained	Mostly in dark
WT-LF	n/a	15	12L:12D, standard 0700-1900 GMT	Restricted, available 0800-2000 GMT	SCN-peripheral alignment disrupted	12 hrs/day in light
Per1/2-AL	10	16	Constant darkness	*ad libitum*	Arrhythmic	Continuous
Per1/2-RF	10	16	Constant darkness	Restricted, available (AS) 2000-0800 GMT (AJ) 0800-2000 GMT	Arrhythmic	12 hrs/ day

Fitness consequences of blood stage accumulation patterns: Experiment 3

The design illustrated in Figure 2.4 tests how well parasites establish the erythrocytic phase following simulated synchronous (Pulse) or asynchronous (Spread) egress patterns. To ensure we did not confound naturally egressing parasites with the experimental *P. chabaudi* infections, we used *P. berghei* to generate an exoerythrocytic phase in all mice. We selected *P. berghei* because it is unlikely to interfere with *P. chabaudi*'s replication during the erythrocytic phase due to a different RBC tropism. All n=35 mice became infected with *P. berghei* (Transmission to infect experimental mice by mosquito bite 16:00-17:00 GMT all treatments), with 5-10 mice per treatment for the experimental *P. chabaudi* introductions over 3 inoculations. Each mouse received a total of $3x10^5$ early IDC stage infected RBC (rings), via intraperitoneal injection in either in a single dose or split into 3 doses (the single dose mice also received 2 injections of PBS carrier), 24:00, 8:00, 16:00 GMT (~47 hours

post mosquito bite). All mice were sampled daily for 5 consecutive days at 8:00 AM GMT by taking 10uL blood for DNA, RBC counts and smears. Because, no sex differences were found, thus we pooled groups in downstream analysis (t = 0.0040, p= 0.9968).

Sampling and data collection

Molecular protocols

Parasites were quantified from 10uL blood samples collected from tail snips with capillaries. We performed DNA extractions using the Semiautomatic Kingfisher machine with the MagMax 96-DNA multi-sample kit (ThermoFisher), following (Schneider et al., 2019, protocol dx.doi.org/10.17504/protocols.io.86fhzbn), with the exception of doubling the sample volume. For each 96-extraction plate, we included positive controls *P. chabaudi* blood stages and water as negative control. After extraction, we performed qPCR targeting gene cg2 by relative standard curve quantification. Plate differences did not affect the qPCR (glm AS F= 0.6641 df= 5, p= 0.655; glm AJ, F= 0.4723, df= 4, p= 0.739). Efficiency was 87.75%, 96.5%, parasite genotype both R^2=0.99, thus we set the same detection limit for each gene to our DNA lowest dilution of the standards, positive controls, 3.5 par/uL (AS) and 5.3 par/uL (AJ).

For sporozoite quantification we carry out procedures as described in (Schneider, Rund, et al., 2018). In brief, we pooled 3 to 6 fed mosquitos to extract DNA as described in (Schneider et al., 2018). After DNA extraction, mosquito sporozoites were quantified by qPCR using 18S primers Bell et al. 2009 (Bell et al., 2009) and PCR conditions, using the TaqMan Fast Universal PCR Master Mix (2x)(Applied Biosystems™), and PCR program as in (14): 95ºC 20 sec, 45 cycles of: 95ºC 3 sec, 60ºC 30 sec (amplification efficiency 90.75% AS, 92.75%, AJ both R^2= 0.99). We included non-infected mosquito DNA extracts and water as negative controls and *P. chabaudi* DNA derived from blood stages as a positive control, using a 1:10 serial dilution series as a reference to quantify parasite genomes.

Statistical analysis

All data analysis was performed using R version 4.0.2 (2020-06-22) packages for analysis (2S Table1) and graphics. We constructed mixed effects models to quantify the relationships between parasites densities and treatments groups following the approach by (Zuur, 2009; Zuur and Ieno, 2016), with mouse identity as random effect to take into account repeated measures on each infection. The response variables were transformed to meet model assumptions of variance homogeneity and normal distribution of residuals: for experiments 1 and 2, we applied elog +1 for liver experiments, and for experiment 3 blood analysis, we used 2log. Model selection was performed *via* model fit, using parametric bootstrap, and log-likelihood ratio test (LRT) in nested models (re-fitted with ML). Model estimates and confidence intervals were computed using Wald statistic for the fixed effects, and denominator degrees of freedom ddf (ddf) noninteger numbers because of mixed effects models were estimated using Kenward Roger approximation. To better describe nonlinear shapes of accumulation patterns, we averaged parasite density across mice within each time per genotype and applied a gam model with a factor smooth (no correlated error structure was considered). Finally, we applied the nonparametric Kruskal Wallis test to compare sporozoite loads across treatment groups, and applied Chi-square test of independence and Friedman test for group by time comparisons with post hoc Wilcoxson pairwise comparisons for cumulative data, and Fisher's exact 2X2 contingency table for comparing independence between sporozoite and hepatocyte numbers.

Results & Discussion

Role of host rhythms in the transition between the exo- and erythrocytic phases

We conducted two experiments to examine whether host TTFL clocks and/or feeding-fasting rhythms influence when the erythrocytic phase is initiated, for two different *P. chabaudi* genotypes (AS and AJ, see methods). Both parasite genotypes experienced perturbations of host TTFL clocks and host feeding-fasting rhythms, as described in Table 2.1. By independently manipulating the duration of infection relative to the timing and presence of the host's feeding-fasting rhythm and TTFL-driven clocks in different treatment groups, our design does not confound a fixed developmental period

coinciding by chance with, for example, the end of the feeding window (Figure 2.1). Thus, by comparing the patterns of accumulation of parasites in the blood across treatment groups, we can assess whether (following a liver phase of ~48 HPI (De Niz and Heussler, 2018) egress is scheduled to align with a particular host rhythm or simply occurs upon completion of a fixed developmental window. Our designs test three hypotheses (Figure 2.1): (i) If egress is timed to align with host feeding-fasting rhythms (hypothesis 1) we predict rapid accumulation in the 3 groups with feeding-fasting rhythms, occurring towards the end of the feeding window (i.e. 12 hours apart in the WT-DL compared to WT-LD / Per1/2-RF groups), but accumulation is gradual in arrhythmic hosts. (ii) If egress is scheduled by host TTFL rhythms (hypothesis 2), its timing is inverted between the WT-LD and WT-DL groups (and must occur at the end of the dark phase for the IDC to begin on time), but in contrast to hypothesis 1, accumulation is arrhythmic in both Per1/2 groups. (iii) If egress occurs after a fixed developmental duration (hypothesis 3), either in a short burst or over a prolonged window, parasites will accumulate following the same pattern across all groups. Note, the treatment groups specified here refer to the experimental design for genotype AS. In the second experiment, using AJ, the same hypotheses apply despite two minor differences in the treatment groups (Table 2.1, Figure 2.1).

We used glmm (generalized linear mixed effect models) using mouse id as random effect to test for treatments and their interactions. We infected mice by mosquito bite to ensure ecological realism of the exoerythrocytic phase, having verified that sporozoite load did not differ between mosquitoes used to infect each treatment group (AS Treatment=χ^2 (3)= 1.71, p= 0.63, AJ Treatment=χ^2 (3)=1.68, p= 0.64). We used a qPCR assay with a detection limit of ~5.7 parasites/μl to quantify parasites in the blood during and beyond the expected window for egress. Following infection by sporozoites, *P. chabaudi*'s exo-erythrocytic phase is estimated to last between 52 to 53 hours (Simwela and Waters, 2022; Stephens et al., 2012) so we began blood sampling at 48 hours post mosquito infection (HPI) and continued until 83 HPI for genotype AS, extending this to 116 HPI for genotype AJ, with the expectation that progeny from the schizogony following completion of the first IDC could contribute to densities from 72 HPI onwards. The detected parasites are most likely IDC stages because merosomes are unlikely to disseminate beyond the lungs (Baer et al., 2007)

although free parasites might occasionally be counted. Parasites in the blood were readily detected by 60 HPI for AS (43% infections patent) and by 48 HPI for AJ (100% infections patent). While parasite densities accumulated in different time-varying manners for both genotypes (Figure 2.2a, 2.2c: AS HPI $F(1, 434.39)= 56.09$, $p <0.001$; AJ HPI $F(1, 434.39)=56.09$, $p <0.001$), parasite density dynamics did not differ significantly between treatment groups for either genotype, this means the slopes are not significantly different (Figure 2.2a,2. 2c: AS HPI:treatment, HPI:Treatment $F(3, 137.78)=2.56$, $p= 0.06$; AJ HPI:treatment, HPI:Treatment $F(3, 431.84)= 2.07$, $p= 0.10$). Furthermore, parasite densities observed over the first IDC (i.e. within 24 hrs of patency) did not differ between treatments (Figure 2.2b, 2.2d: AS $F(3, 16)= 0.39$, $p= 0.76$; AJ $F(3, 56.84)= 0.84$, $p= 0.47$). Thus, both the pattern of accumulation of blood stage parasites and their productivity are independent of host feeding-fasting and TTFL rhythms. Instead, accumulation in the blood following a fixed developmental period in the liver explains the patterns (hypothesis 3; Figure 2.1). Despite some egress clearly occurring before previously published durations for the exoerythrocytic phase, especially for genotype AJ, AJ parasite densities at 48 HPI do not differ between treatment groups ($\chi^2(3)= 3.00$, $p= 0.39$). Whilst one AS infection (Per1/2-RF) was patent at 48 HPI, sensitivity analysis reveals no treatment group differences whether including (Treatment $F(3, 127.00)= 1.36$, $p= 0.258$) nor excluding (Treatment $F(3, 124.77)= 1.2402$, $p= 0.298$) this data point. It is also unlikely that the treatment groups followed different egress schedules during the gap between the first (48 HPI) and second (60 HPI) sample for AS (Figure 2.2a). This gap is only 12 hours and so, any impact of host rhythms would be evident in the samples from the subsequent half of the host's circadian cycle (60 HPI). Furthermore, to have missed treatment group differences for AJ, this genotype would have to begin egress 24 hours after entering the host, which is far earlier than documented for any *Plasmodium spp*.

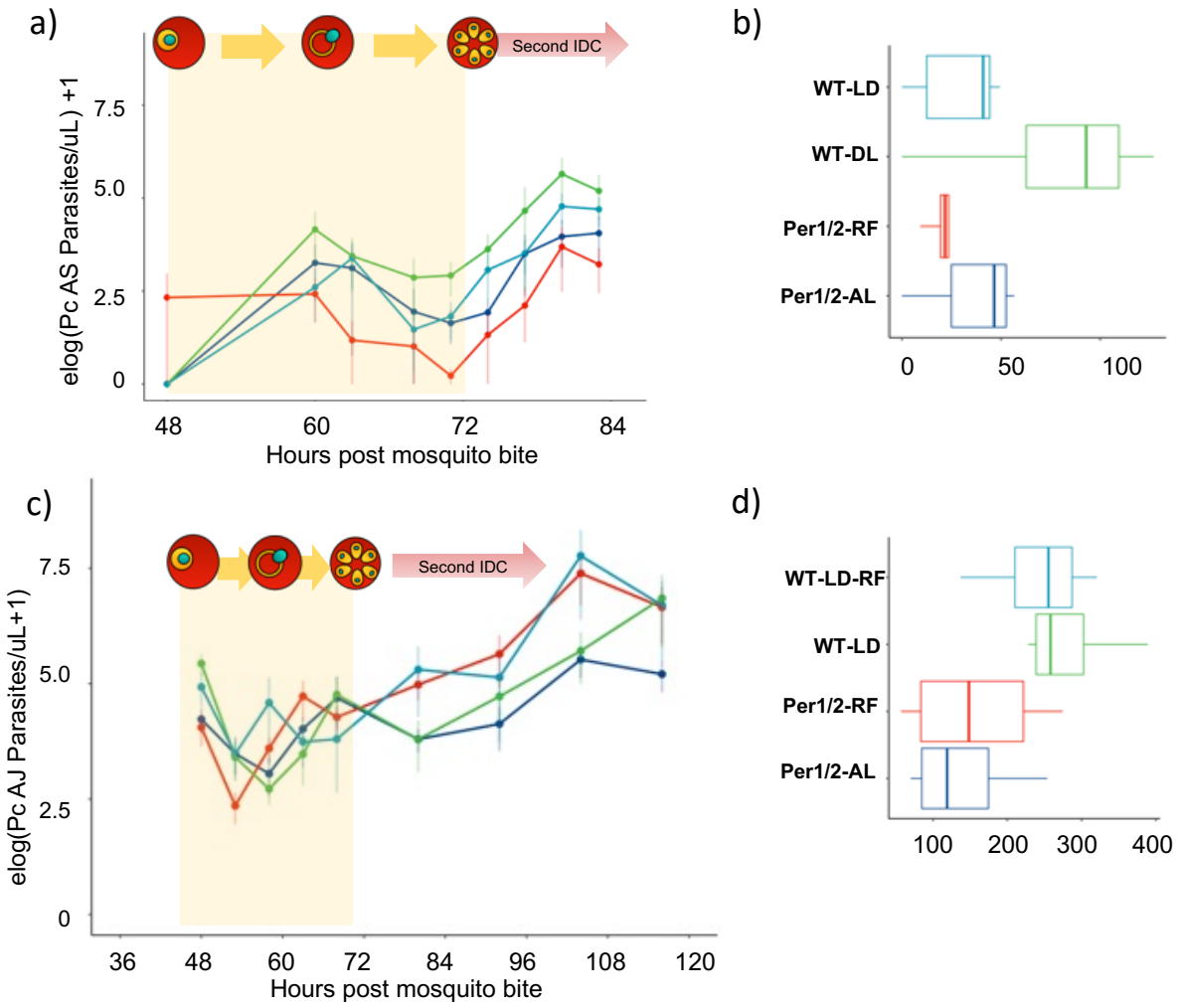


Figure 2.2. Parasite densities in peripheral blood following egress from the exo-erythrocytic phase. Mean +/- SEM parasite density dynamics for AS (a) and AJ (c), with yellow shaded region indicating when parasites are within the first IDC assuming a 24 the first IDC. Treatments AS (a,b) and AJ (c,d) WT-LD as described in Table 1. Exo-erythrocytic phase of ~48 hours (Stephens et al., 2012), after which, replication within the erythrocytic phase likely contributes to parasite densities. Interquantile ranges (IQR) for AS (b) and AJ (d), for cumulative parasite densities first IDC. n=10 see table 2.1 for sample size details.

There are several non-mutually exclusive explanations why, unlike for IDC stages, host rhythms do not affect *Plasmodium* parasites transitioning from the liver to the blood (Box 2.1). First, the liver is a nutrient rich environment, which when coupled with *Plasmodium's* ability to manipulate hepatocyte nutritional content and clock mediated processes (i.e., autophagy), may compensate for the parasites inability to store essential nutrients to ensure sufficient resources to support replication around the clock (Prado et al., 2015). In contrast, IDC stages experience limitation for nutrients (such as glucose and/or isoleucine) during the host's fasting phase (Hirako et al.,

2018; Prior et al., 2021). Second, if replication within hepatocytes is arrhythmic or occurs in cycles much faster than the IDC, demand for resources may be evened out across the host's circadian cycle. Our results suggest that parasites do not experience such constraints, suggesting that observations of heterogeneity in productivity between infected hepatocytes (Afriat et al., n.d.), is better explained by stochastic processes rather than rhythmicity in resource limitation. A third explanation is that perhaps despite the liver environment being rich in time-of-day information, exo-erythrocytic stages are unable to detect or respond to the signals that set their IDC schedule (O'Donnell et al., 2022; Prior et al., 2020). This might reflect a trade-off; if parasites are only able to follow rhythms if they have a duration of 24 hours, it might not be possible for the small number of sporozoites that successfully invade hepatocytes to be productive enough within the approx. 2 day window of the exo-erythrocytic phase.

Genotype-specific patterns of accumulation following egress

The window for transitioning between the liver and blood spans differs between species. In *P. yoelii* release of merosomes spans over ~10 hours (Baer et al., 2007), from 46 to 56 hrs since infection, and this occurs over a window of 6 hours for *P. berghei* (De Niz et al., 2016), from 36 to 42 hrs post infection. Our estimated liver stage duration for *P. chabaudi* is 48-60 hrs, which is slightly shorter than previous estimates for *P. chabaudi* (~52-53 h) and positions it between the predicted windows for *P. berghei/P. yoelii* (~43-50 h) and for *P. vinckei* (~ 53-61 h) (Stephens et al., 2012). Therefore, our accumulation patterns (Figure 2.2) are consistent with the liver stage duration for *P. chabaudi*.

Parasite dynamics (Figure 2.3) demonstrate that AS and AJ exhibit significantly non-linear egress patterns (AS sHPI, edf= 3.206, ref.edf= 3.851, F=10.048, p=<0.001; AJ sHPI: edf=3.827, ref.edf= 4.392, F= 6.393, p= <0.001), and these patterns are different between genotypes (Genotype, F= 50.81, p= 3.6e-12) Given the challenges of investigating exo-erythrocytic stages and early IDCs, genetic variation in the processes and host-parasite interactions involved have not previously been tested. For AS, half of infections are patent in the blood between 48-60HPI suggesting a long

window for egress. The first IDC stages will begin to sequester (withdraw into capillaries; (De Niz et al., 2016) from about 60 HPI, and so, if egress and sequestration are simultaneously occurring, this could explain the dampened rhythm for AS densities in the first 24 hours, with the progeny of the earliest egressed parasites driving the rapid rise from 72 HPI. In contrast, that almost all AJ infections were patent in the blood by 48-53 HPI suggests AJ egresses earlier than AS. Earlier egress should constrain the window available for e.g. sequestration which is reflected by AJ dynamics being more synchronous (O'Donnell et al., 2013) than observed for AS. Genotype specific patterns are not explained by different number of sporozoites being inoculated by mosquitoes. Instead, we propose there is genetic variation in how likely sporozoites are to establish infections within hepatocytes. Following (Bejon et al., 2005), assuming 2 ml of blood per host, an infected hepatocyte yielding a minimum of 10,000 infected RBC, and extrapolating from the peak densities measured within the first IDC, we estimate that AS infected ~5.52 hepatocytes / host, whereas AJ infected ~24.47 hepatocytes this difference is not driven by variation in sporozoite inoculum (Fisher's exact test p = 0.4166). Thus, mirroring that AJ reaches higher densities in the blood than AS (Antia et al., 2008), AJ also appears more productive in the liver. It is not known if the strain AJ is more productive during the liver or possesses a higher infection rate of hepatocytes.

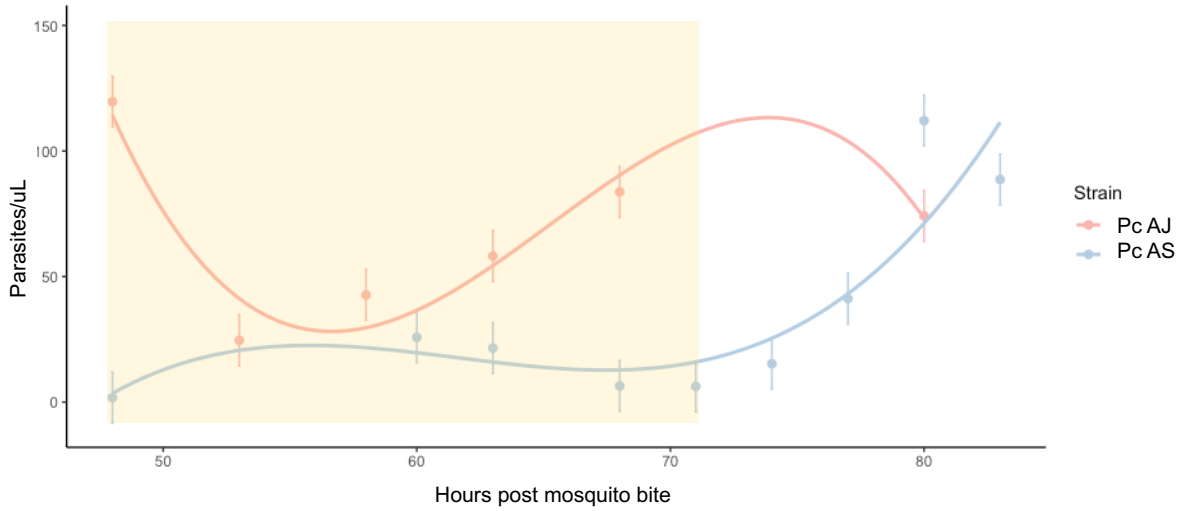


Figure 2.3. Nonlinear patterns of accumulation in the blood following the exo-erythrocytic phase with yellow shaded region indicating when parasites are within the first IDC assuming an exo-erythrocytic phase of 48 hours (De Niz and Heussler, 2018). From the assumed peak of first IDC, we used the average number at peak to estimate hepatocytes infected, like in (Bejon et al., 2005).

The patterns of accumulation (Figure 2.3) represent the net outcome of egressed parasites that have invaded RBC and successfully initiated the first IDC, offset by dead/ killed parasites released from unviable merosomes that are rapidly cleared, sequestration of late IDC stages, as well as stochasticity associated with detecting low densities and sampling error. These processes could vary between genotypes for a number of non-mutually exclusive reasons and mean that the liver productivity estimates above are cautious. First, genetic differences underlying RBC invasion genes, in which AJ is predicted to be able to infect a broader RBC age range than AS, may contribute to AJs higher densities at the start of the sampling window (Antia et al., 2008). Second, AJ is impaired for sequestration (Nahrendorf et al., 2021) so will be present in higher densities than AS at late IDC stages. Potentially, a shorter window for egress correlates with stronger immune stimulation, resulting in a greater loss of number for AJ as the first IDC progresses. Third, different experiments are expected to exhibit different sampling errors, potentially introducing sources of heterogeneity at different levels in AS and AJ densities. However, noise can be parsed out by appropriate statistics and does not generate bias, giving confidence in the conclusion that different patterns are exhibited for each genotype. Intraspecific variability within the exo-erythrocytic phase remains poorly explored, yet our data suggest they correlate with the genetic differences that manifest in the erythrocyte phase (Grech et al., 2006; Schneider, et al., 2018), raising new questions (Box 2.1). Answering these questions may reveal that the exo-erythrocytic phase is not clinically silent and open up novel intervention strategies.

Fitness consequences of blood stage accumulation patterns

Given the importance of aligning the timing of the IDC with host rhythms and that misaligned parasites actively reschedule the IDC, our finding that the erythrocytic phase is initiated in a manner independent of host rhythms was unexpected. Whilst parasites only require approximately 6 IDC to change their timing by 12 hours, spending any number of IDCs misaligned to host rhythms imposes fitness costs (O'Donnell et al., 2011; O'Donnell et al., 2022) This suggests that during the transition from the exo-erythrocytic to the erythrocytic phase, a constraint may render parasites unable to detect or respond to time of day information, enforcing a strategy that is

suboptimal for fitness. For example, stochastic processes could influence how long merosomes spend in the lungs before release (Baer et al., 2007), as well as a wide distribution of egress times being generated by an unavoidable time lag between breaking out of the inner membrane PVM (parasitophorous vacuole membrane) and the cell derived membrane. Alternatively, accumulating in the blood in a gradual manner might be advantageous by, for example, minimising the risk of synchronous egress stimulating dangerous immune responses.

We tested whether the manner the erythrocytic phase is established interacts with host rhythms to impact on parasite fitness, using the replication rate of the first 5 IDCs as a proxy for the within-host survival component of fitness. Specifically, we experimentally simulated initiation of the erythrocytic phase occurring as parasites entering the blood in a single "pulse" (WT-Pulse, Per1/2-Pulse, Per1/2-Pulse+8) or "spread" (WT-Spread, Per1/2 -Spread) equally over 3 injections spanning a window of 16 hours, in both wild type and Per1/2-null mice (Figure 2.4). First, we infected all mice with *P. berghei* by mosquito bite to establish an exoerythrocytic infection before introducing experimental *P. chabaudi* parasites and using a species-specific qPCR to follow the densities of *P. chabaudi* parasites in a manner unconfounded by any *P. berghei* egress. By introducing experimental parasites into mice with an ongoing exo-erythrocytic infection, albeit of a different species, we facilitated ecological realism for incoming parasites by allowing host physiology and immune response to respond to liver stage parasites. All infections were sampled daily for 5 days starting 24 hours after the 2nd injection to quantify *P. chabaudi* densities in the blood and compare replication rates. Our design enables the impact of spread versus pulse egress strategies to be compared (within each type of host), as well as testing whether host rhythms affect the consequences of each strategy (by comparing each strategy between WT and Per1/2 hosts), and comparing the Per1/2-Pulse with another group in which parasites were introduced 8 hours alter (Per1/2-Pulse+8) to test whether the duration of time since initiating the liver phase matters (e.g. by allowing stronger immune responses that affect IDC stages to develop).

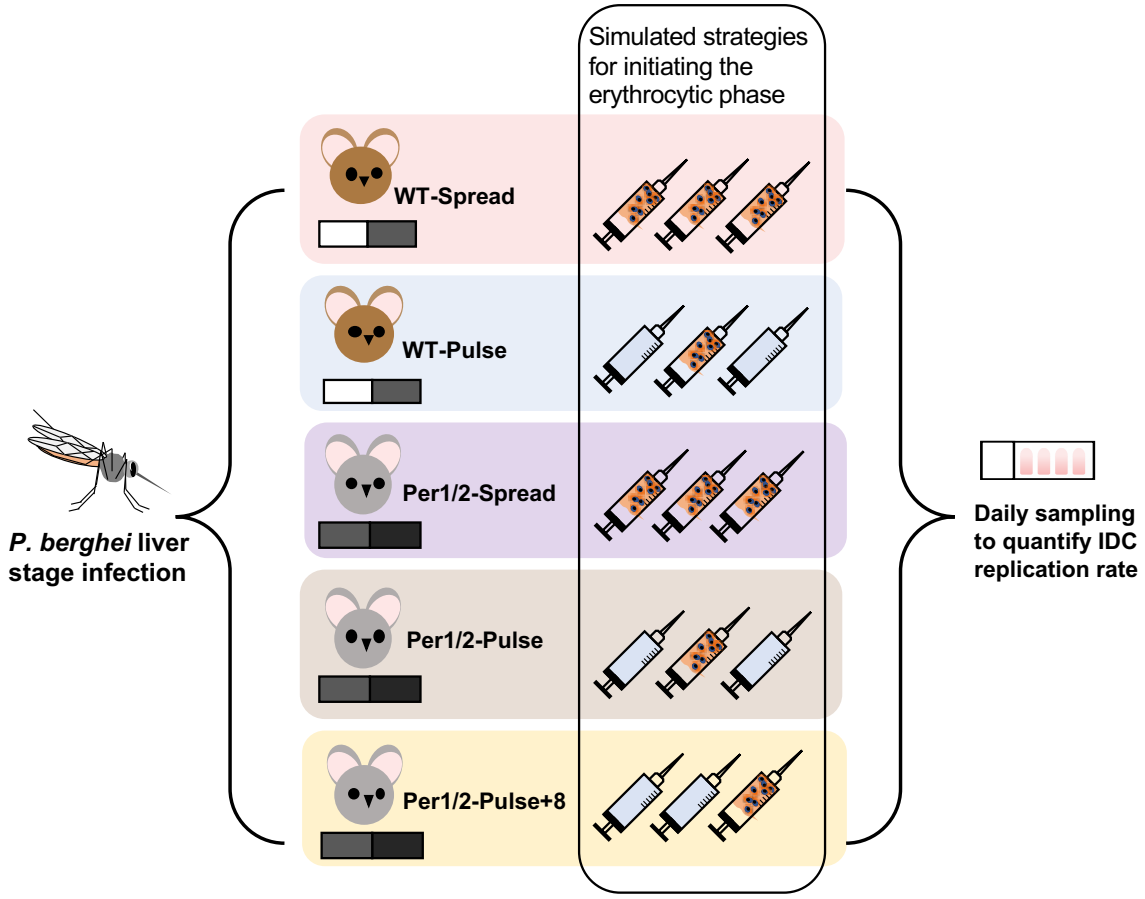


Figure 2.4. Experimental design to test whether different strategies for initiating the erythrocytic phase and host rhythms impact on within-host fitness. Hosts were either wild type C57BL6 (WT, n=20) kept in standard (WT with functional TTFL and feeding-driven rhythms, or *Per*1/2-null mice with a disrupted TTLF (n=15) housed in constant darkness to ensure arrhythmicity in feeding-fasting and other rhythms. Prior to the experiment all mice were acclimated to their photoschedules and then infected by mosquitoes harbouring *P. berghei* sporozoites to generate a liver-phase infection prior to introducing P. chabaudi into as ecology realistic conditions as possible, but in a manner that allows species specific assays to discriminate the performance of the focal *P. chabaudi* parasites from any egressed *P. berghei* in the blood. At 31 HPI, just before *P. berghei* is expected to egress, each host received 3 injections 8 hours apart in which 3x10^5 red blood cells infected with ring stage *P. chabaudi* AJ were introduced directly into the blood split equally over the 3 inoculations ("spread": to represent the patterns observed in our first experiments), or in a single dose ("pulse": to represent the IDC initiated by a synchronous pulse aligning to the time of day the IDC is naturally at ring stage) with PBS carrier given in the 2 placebo injections. All infections were sampled daily for 5 days starting 24 hours after the 2nd injection to quantify *P. chabaudi* densities in the blood and compare replication rates. This design enables the impact of spread versus pulse strategies to be compared (within each type of host) as well as testing whether host rhythms affect the consequences of each strategy (by comparing each strategy between WT and Per1/2 hosts), and comparing the Per1/2-Pulse and Per1/2-Pulse+8 (pulse delayed by 8 hrs) groups to test whether the duration of time since initiating the liver phase matters (e.g. by allowing stronger immune responses that affect IDC stages to develop).

We expected the patterns of accumulation we observed (Figure 2.2) to be a constraint and that the best strategy would be to begin the erythrocytic phase in a synchronous burst aligned with the host's feeding-fasting rhythm (i.e. parasites in WT-pulse and Per1/2-pulse hosts perform best). Instead, parasite densities increased monotonically following near-identical trajectories across all treatment groups (Figure 2.5), (slope comparison: LRT, $\chi^2(4)= 0.304$, p= 0.98, Treatment $\chi^2(4)=8.36$, p= 0.091), following the linear equation of 2Log(Parasites/uL)= 5+2.48Day. This translates to a 5.6 fold per IDC increase. Furthermore, the cumulative totals do not differ between treatments ($\chi^2(4)= 6.7947$, p= 0.147). Thus, neither host rhythms, the duration of the exo-erythrocytic phase, nor the manner in which the erythrocytic phase is initiated affect within-host replication.

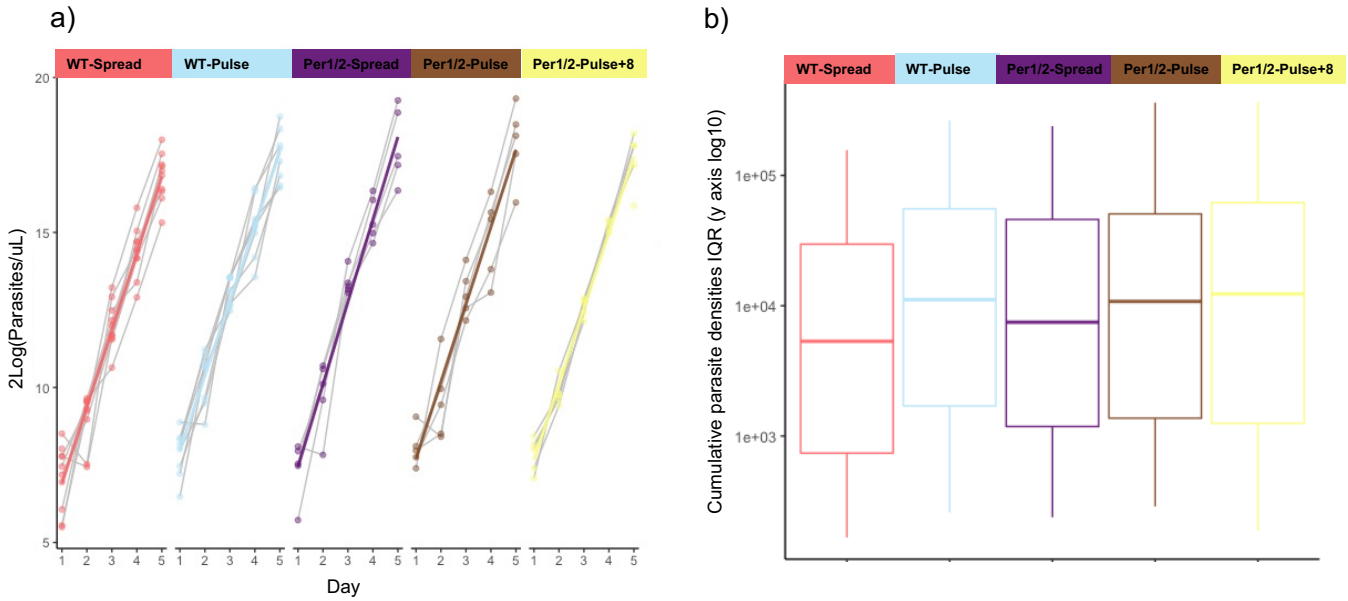
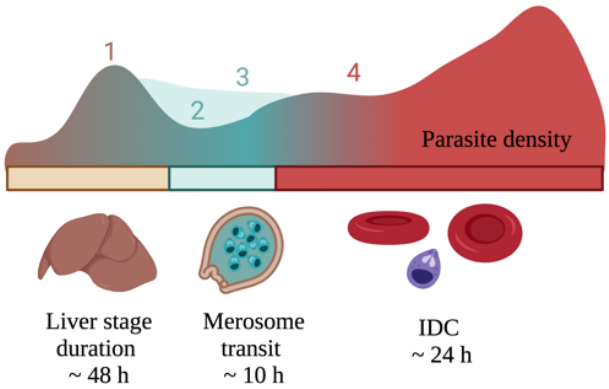


Figure 2.5. Neither simulated egress strategies, host rhythms, or duration of exo-erythrocytic phase affect the establishment of parasites in the blood phase. Parasite densities for each infection represented by dots connected by grey lines, with the mean linear trend colored according to treatment group (a). IQR cumulative parasite densities per treatment, colours indicate treatment groups, WT-Spread (pink), WT-Pulse (blue), Per1/2 Spread (purple), Per1/2 Pulse (brown) and Per1/2 Pulse+8 (yellow)(b). n= 5 -10.

Our results suggests that liver egress strategies are selectively neutral and so, it does not matter which strategy parasites evolve. However, because *Plasmodium* has the ability to tell the time (O'Donnell et al., 2020; Prior et al., 2020; Subudhi, et al., 2020), and if this involves a circadian oscillator (Rijo-Ferreira et al., 2020), the default expectation is that all stages in the lifecycle are constitutively rhythmic. As discussed

above, decoupling exo-erythrocytic stages from a circadian clock may release parasites from a constraint of only being able to replicate at intervals of 24 hours. Such flexibility could come from using a simpler “just in time” strategy in which they respond directly (plastically) to timing cues rather than an endogenous clock (Prior et al., 2020). Evolutionary theory for plasticity suggests that the maintenance of mechanisms to sense the environment and process information requires resource expenditure (DeWitt et al., 1998).Thus, it is possible that exo-erythrocytic parasites also save on the costs of generating and operating environmental sensing mechanisms when there are no fitness gains to be made from scheduling their activities. This scenario assumes that *Plasmodium* has evolved the ability to efficiently establish the correct IDC schedule once in the blood to minimise the window during which some parasites are misaligned to host rhythms raising questions regarding fitness benefits of adaptive plastic IDC from liver stage, and new directions to explore (Box 2.1). Furthermore, the within-host benefits of a correctly scheduled IDC are associated with acquiring resources that may become limiting as infections progress and hosts become sick. Thus, parasites at the start of the erythrocytic phase are more likely to have sufficient resources to exploit around the clock, which minimises any costs of misalignment within the first few IDC.

Box 1



1) Replication within hepatocytes
- Is replication unconstrained by resource availability?
- Can parasites entrain in the liver?
- Does productivity and developmental duration vary between genotypes / species?
- Does gametocyte conversion vary and affect productivity of asexually committed merozoites?

2) Merosome egress and travel to organs
- Do merosomes vary in their contents, where they burst and how long they spend there, within and between genotypes/ species?

3) Merozoite egress directly from hepatocytes
- What proportion of IDC stage parasite derive from this route? Does this vary between genotypes / species?

4) Establishing the IDC
- Does RBC invasion gene expression vary within and between genotypes?
- Do different egress patterns stimulate different risks of immune responses?
- How do parasites alter IDC phenotypes to align blood stage replication with the hosts feeding-fasting rhythm?

Box 2.1. Outstanding questions and working model of parasite accumulation after liver emergence.

Conclusions

Our experiments, using two genetically distinct parasite strains, reveal that the accumulation of parasites in the blood following the exo-erythrocytic phase occurs over a prolonged window, following a fixed developmental time in the liver. That host rhythms, especially feeding-fasting rhythms, do not influence the timing of initiating the erythrocytic phase (Figure 2.2) was unexpected because exo-erythrocytic parasites are considerably more metabolically demanding than IDC stages (Zuzarte-Luis & Mota, 2020) and many rhythmic hepatocyte processes (including autophagy, detoxification, cholesterol levels, and lipid synthesis) are expected to affect the rate and so, timing, of parasite development (Zwighaft et al., 2016). However, compared to red blood cells in which IDC progression is influenced by the rhythmic availability of resources from host food, hepatocytes are far better resourced and may be able to support far higher parasite burdens around the clock. Furthermore, liver stage parasites are able to manipulate hepatocyte autophagy to facilitate their development, and so, may be able to manipulate other processes such as the hepatocyte clock to ensure continuous access to resources and dampen dangerous autophagy activities by lysosomes (Schroeder et al., 2022).

We also note that the exo-erythrocytic developmental window of parasites in some treatment groups (AS, WT-DL; AJ, WT-LD) encompassed an additional feeding window compared to other groups (AS, WT-LD; AJ WT-LF, Per1/2-TRF). Yet, having greater access to a range of resources from the host's food, including fatty acids (Kaushansky and Kappe, 2015), phosphatidylcholine (Itoe et al., 2014), phosphoinositol (Thieleke-Matos et al., 2014), amino acids (e.g. arginine) (Zuzarte-Luis and Mota, 2020), iron (Zuzarte-Luis and Mota, 2020), and particularly resources such as cholesterol that cannot be stored (Coppens, 2013), did not enhance parasite productivity (Figure 2.2) further emphasising that host metabolic rhythms are not limiting for exo-erythrocytic stages. One caveat is that our experiments used well fed, healthy, adult mice as hosts and so, host feeding-fasting rhythms may become relevant to exo-erythrocytic stages and their transition to the blood in wild hosts in poor condition due to environmental stressors.

A limitation of our design is that because egress cannot be measured directly *in vivo*, we assume that the accumulation of blood stage parasites reflects the timing of

egress. For example, it is possible that egress of merosomes from the liver does follow a specific schedule that happens to be obscured by high variation in how long merosomes spend in tissues. Even if this is the case, gradual accumulation to initiate the erythrocytic phase remains the important phenotype, because this is what natural selection is able to act on, and this is arrhythmic. Our final experiment reveals that gradual accumulation is not a target of selection because IDC stages replicate equally well, however and whenever they appear in the blood (Figure 2.5).

Compared to the erythrocytic phase, the ecology of the exo-erythrocytic phase remains a black box. Here, we reveal that timing does not play a role in parasite-hepatocyte interactions underpinning the transition to the erythrocytic phase. Thus, we propose that *Plasmodium's* ability to alter the timing of the IDC has evolved to ensure blood stage replication quickly becomes correctly aligned to the host's feeding-fasting rhythm (O'Donnell et al., 2020, 2021). More broadly, our finding suggests that disrupting parasite time-keeping will not interfere with egress from the liver, but could prevent parasites from establishing a successful blood stage infection, reducing both pathology and transmission.

Chapter 3. Do host rhythms affect parasite sequestration during blood-stage replication?

Abstract

Plasmodium parasites withdraw from the peripheral circulation to avoid clearance in the spleen by "sequestering". Sequestration involves parasitised red blood cells adhering to endothelial cells in various tissues and organs. Little effort has been made to understand the ecological and evolutionary relevance of this parasite strategy. For example, the costs and benefits of sequestration are not well quantified and whether sequestration rates vary according to host or parasite factors is poorly understood. Here, I explore whether host daily rhythms represent an unexplored physiological characteristic that mediates daily fluctuations in the rates and benefits/costs by sequestration. Asexual stage parasites sequester during late developmental stages and because development is linked to host daily rhythms, sequestration is therefore also rhythmic. In this study, I explore whether host rhythms – that are either synchronised to the light-dark cycle and/or to feeding-fasting rhythms – affect a proxy for levels of sequestration and the consequences for parasite replication. We discovered that parasite sequestration in the liver and the lungs is complex and correlates with the interaction of multiple host rhythms, whereas host rhythms do not affect parasites sequestering in the spleen. However, these complex ecological patterns do not impact on parasite replication. While parasite replication causes anaemia, sequestration is directly responsible for a range of severe disease manifestations, such as cerebral complications, coagulation and embolism (Rogerson, 2003). Thus, our results suggest interrupting host rhythms might alter the abundance of sequestering parasites within tissues, limiting a range of pathologies.

Introduction

Like all parasites, *Plasmodium* parasites (which cause malaria), have evolved a suite of behaviours to counter and/or evade unfavourable host responses to infection. For example, *Plasmodium*, like trypanosomes, modify the antigens they expose to the host throughout infections (Schmid-Hempel, 2021). Such antigenic variation diverts host

immune responses to the most frequent variant encountered, leaving less frequent variants to persist and replicate; consequently, generating cycles of frequency-dependent parasite variants during infections. Another strategy, present in *Plasmodium* spp., assumed to facilitate immune evasion is 'sequestration' (Roberts et al., 1993). *Plasmodium* parasites replicate asexually in the blood of their vertebrate hosts and this intraerythrocytic development cycle (IDC) requires parasites to transition through distinct developmental stages. Later IDC stages sequester from the circulation by attaching to endothelial cells in peripheral vessels within organs. This helps parasites to evade clearance during transit through the spleen because having reduced the elasticity of the red blood cells (RBC) they reside within as an unfortunate consequence of growth, their ability to pass through endothelial slits in the spleen is reduced (Suwanarusk et al., 2004). In contrast, early IDC stages can remain in the circulation because they are yet to affect RBC elasticity and so, are less vulnerable to splenic clearance. Other benefits of sequestration may include locating to specific tissues that provide the optimal microenvironment for growth, and to locations from which parasites can enhance survival through immunomodulating actions (Sherman, et al., 2003). However, the benefits of sequestration may be offset by costs incurred in the deployment of sequestration behaviours; when the selective drivers are absent (i.e. the host is splenectomised or in culture conditions), parasites' ability to sequester diminishes, suggesting sequestration is intrinsically costly (Bachmann et al., 2009).

Understanding the costs and benefits of sequestration is further complicated by this trait being a product of activities of both the host and the parasite. For example, host factors including nutritional state (Frankland et al., 2007), temperature (Rawat et al., 2021), shear stress (Nyarko et al., 2020), and immune responses (Brugat et al., 2014) alter the expression of *Plasmodium* exposed Ag (Antigens) which are used for sequestration. Different *Plasmodium* spp. preferentially sequester at different sites (i.e. demonstrate different tropisms) within the host and begin to sequester at different points in the IDC (Simwela and Waters, 2022). This is partially because ligands used by different parasite species possess different affinities to host receptors, for example, *P. falciparum var2csa* generates the placental sequestration phenotype, mediated through binding to host chondroitin sulphate A (CSA)(Serghides et al., 2006). Some of the parasite's ligands known to bind to different host receptors include PfEMP1,

RIFIN, STEVOR, PIR, and host receptors include CD36, CSA, ICAM1, and VCAM1 (De Niz and Heussler, 2018; Lee et al., 2019).

That variation in sequestration patterns is linked to both parasite and host factors indicates the ecology of sequestration is intricate and may also have complex relationships with parasite fitness. Other complexities may include whether the host imposes any constraints on parasites' ability to sequester. For example, at high infection intensities, perhaps sequestration sites become crowded and parasites compete for host receptors to bind to, potentially selecting for sequestration earlier in the IDC or for tropism to less desirable sites. Furthermore, host circadian clocks drive rhythmicity in the expression of around ~40% of mammalian protein-coding genes that oscillate in at least one organ (Zhang et al., 2014), including receptors involved in sequestration, like ICAM-1, VCAM-1 and CD36. For example, ICAM-1 is expressed in all capillary beds and its expression peaks during the active phase for mice, which is in the evening (He et al., 2018). Host rhythms also influence leukocytic trafficking (He et al., 2018; Pick et al., 2019), intrinsic cellular immune activity (Ohkura et al., 2009; Pritchett and Reddy, 2015), as well as the nutritional content, temperature, and oxygen tension of blood (Reinke and Asher, 2019; Rodrigo and Herbert, 2018). These rhythms could interact to affect sequestration opportunities and alter the costs and benefits of the sequestration by contributing to variation in the microenvironment that sequestering parasites experience (Figure 3.1). Host rhythms are more likely to influence sequestration opportunities and its consequences for parasites with a rhythmic than an arhythmic IDC. For species with a rhythmic IDC, asexually replicating parasites within each IDC cohort develop in synchrony with each other and transition between IDC stages at certain times of day (Mideo et al., 2013; Prior et al., 2020). Most *Plasmodium* spp. including the most virulent human malaria parasite *P. falciparum,* exhibit synchronous IDCs. Indeed, the interval between periodic bouts of fever caused when parasites burst at the end of the IDC to release their progeny was used as a diagnostic feature of malaria infection (Garcia et al., 2001). *Plasmodium* spp. with a rhythmic IDC have an opportunity to align the need to sequester with the timing of when it is most beneficial; when receptors peak, or the microenvironment is best.

Studying how host and parasite rhythms shape sequestration cannot ethically be examined in humans because it is so invasive, and studying human parasites in culture is not a solution due to absence of rhythms and organs. Rodent malaria models offer an ecologically relevant *in vivo* setting for sequestration studies but unfortunately, the main rodent models - *P. berghei* and *P. yoelii* - exhibit an asynchronous IDC. In contrast, the rodent malaria model *P. chabaudi* is rhythmic, with an IDC lasting approx. 24 hours. *P. chabaudi's* IDC is scheduled to align with the host's feeding-fasting rhythm (Hirako et al., 2018; O'Donnell et al., 2020; Prior et al., 2018). Specifically, the IDC is completed during the feeding window when essential nutrients (such as isoleucine) are made available in a rhythmic manner from the host's digestion of its food (Prior et al., 2021). The critical role of ICAM1 for *P.chabaudi* was demonstrated by Cunningham et al., 2017, in contrast to knockout mice lacking CD36, mice lacking ICAM1 have a higher proportion of circulating schizonts compared to wild-type mice(Cunningham et al., 2017). In addition to endothelial receptors, *P. chabuadi* tissue specific sequestration has been described to change between days, initially being highest in the spleen (but confounded due to lack of perfusion in Brugat et al., 2014), followed by the liver and finally in the lungs suggesting host driven factors (i.e. immune response) altering important sequestration factors for the parasites (Brugat et al., 2014). However, whether daily oscillations matter for sequestration/accumulation has not been explored within a day. Given the potential for host rhythms to alter both the microenvironment (via nutrient availability amongst other factors) and the availability of sequestration receptors, we used *P. chabaudi* to test whether host rhythms influence sequestration patterns and their consequences for parasite replication.

Due to the lack of knowledge about the ecology of sequestration and the complexity of interactions between host and parasite rhythms, we made no specific *a priori* hypothesis, but did anticipate some general findings based on the ecology of IDC stages and general patterns of murine daily rhythms (Figure 3.1). First, sequestration rates might be higher in the host's active phase (i.e. night time for nocturnal mice) simply because this is when the expression of genes coding for sequestration receptors peaks. Second, sequestration might be most beneficial in the active phase because parasites move to a less oxidatively damaging environment than in the circulation (but see (Taylor-Robinson, 2000)). However, the liver might be an exception to this pattern because it is responsible for oxidative stress generated by

metabolism. In this case, aligning sequestration with rhythms in feeding-fasting might be more important than aligning with photoschedule-driven host rhythms, at least for sequestration in the liver if not host-wide. Third, the activity of circulating T and B immune cells (Wang et al., 2022), phagocytosis, platelet activity and cytokine production are highest in the active phase (Ohkura et al., 2009; Pritchett and Reddy, 2015). Thus, if the circulation is a more dangerous place than the tissues during the active phase (due to either or both oxidative stress and immune cells), sequestration in the active phase brings the most benefits. However, it is unclear to what extent rhythms in immune responses - as well as rhythms receptor availability - are driven by peripheral oscillators (which are strongly influenced by feeding-fasting rhythms) and/or the Suprachiasmatic nucleus (SCN) which uses light as a time cue (Zeitgeber). This makes it difficult to predict whether aligning sequestration with host feeding-fasting or with photoschedule-driven (i.e. SCN-driven) rhythms provides parasites with the most opportunities to sequester and the greatest benefits.

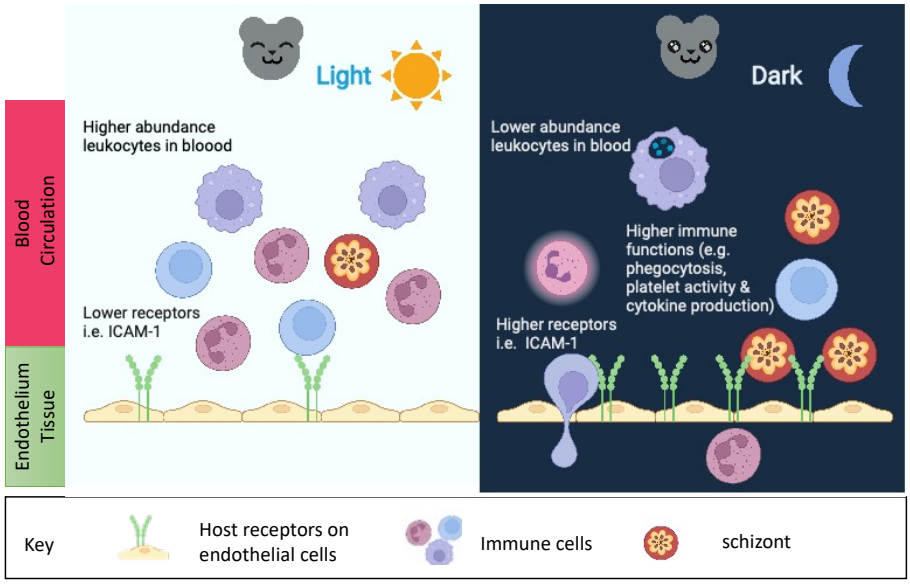


Figure 3.1. Cartoon representation of the different within-host conditions that parasites at the sequestering stage of the IDC (i.e. schizonts) experience during different phases of the host's daily rhythms and putative consequences for sequestration opportunities. For example, in the rest phase (Light), fewer parasites may sequester (bind to endothelial receptors) because less sequestration ligands are available and immune cells are more abundant in the circulation (albeit less active), than in the active phase. Diagram modified from (Reglero-Real et al., 2019). and created using Biorender.

To tease apart to what extent host rhythms affect sequestration we introduced parasites at an IDC stage prior to sequestration into hosts with different perturbations to their feeding-fasting and SCN-driven rhythms, so that parasites experienced

different host times of day with respect to these rhythms, and hosts with/without internal disruption to their rhythms. By following the activities of the experimental parasite cohort, we directly assess the impact of host rhythms and their timing ("phase"). This design means that sequestration is not confounded by the longer term influence of host rhythms on parasite replication and/or the IDC schedule (Greischar et al., 2019). We also examined the consequences of sequestration activities for the replication rate of the experimental cohort. Our study opens the black box of the evolutionary ecology of sequestration which is an important trait to understand because variation in the tissues that different *Plasmodium* species/genotypes sequester correlates with their virulence (Lin et al., 2018) and underpins pathogenesis (Brugat et al., 2014; Lin et al., 2017). Thus, disrupting sequestration could reduce the severity of disease symptoms, and the success of such strategies could be facilitated or hindered by ecological factors such as host time of day.

Methods

Mice and parasites

We used a mix of male and female C57BL6 mice at 8 -12 weeks as hosts, housing them at 21°C in a 12h light: 12h dark photoschedule, with a standard RM3 pelleted diet (801700, SDS, UK) and unrestricted access to drinking water supplemented with 0.05% para-aminobenzoic acid (Jacobs, 1964). We entrained mice to their respective light-dark photoschedules and feeding-fasting schedules for at least 15 days before infection. For feeding-fasting schedules, some treatments involved restricting access to food for 12 hours each day. Such 'timed restricted feeding' does not cause caloric restriction and so did not affect mouse weight (repeated measurements anova, $F(2, 58)= 1.369$, $p = 0.26$).

We infected mice with *Plasmodium chabaudi* AS because previous observations on sequestration patterns and tissue distribution dynamics (Brugat et al., 2014) for this genotype provides a baseline for our study. Parasites were revived from frozen stock and expanded in donor mice (lights on from 22:00-10:00 GMT with *ad libitum* access to food), to generate synchronous infections from which parasites were harvested on day 5 post-infection. We harvested parasites at the end of the light phase (Time since

lights on: Zeitgeber Time, ZT 10-13), when they were at the mid-late trophozoite stage, which is prior to the schizont stage when sequestration predominantly occurs (Cunningham et al., 2017). Trophozoites harvested from 5 mice were pooled, and injected intravenously into experimental mice, at a dose of $2x10^7$ parasitised RBC/mouse. All procedures were carried out in accordance with the UK Home Office regulations (Animals Scientific Procedures Act 1986; SI 2012/3039) and following ethical review at the University of Edinburgh.

Experimental design

To test the impact of the hosts' photoschedule (i.e. SCN)-driven rhythms and feeding-fasting rhythms on parasite sequestration, we created four treatment groups (Figure 3.2). Our treatments cross-factor whether the parasites' IDC schedule is aligned/misaligned to SCN-driven and/or feeding-fasting rhythms. We achieved misalignment by reversing the hosts' photoschedule compared to that of the donor mice (i.e. groups 3, 4) and/or by restricting food to the light phase (ZT0-12) (i.e. groups 2, 3). Our four treatment groups were as follows:

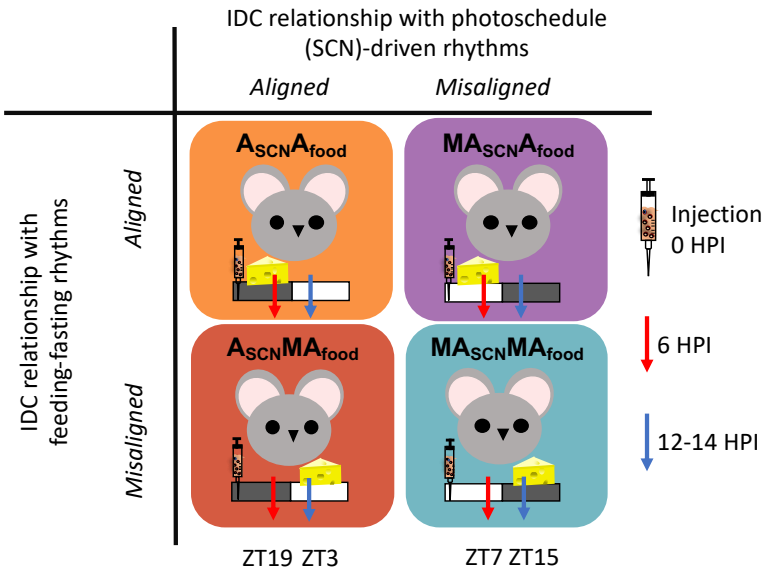


Figure 3.2. Experimental design and sampling. We introduced parasites by intravenous injection prior to the onset of sequestration into hosts experiencing different schedules for their feeding-fasting rhythms (12 hour long feeding windows denoted by cheeses) and their photoschedule (SCN) driven rhythms (denoted by 12 hour light – 12 hour dark bars). The design aimed to force parasites to sequester at different host times of day (phases) according to host feeding-fasting and SCN driven

rhythms, and to sequester in hosts whose rhythms are in synch or disrupted. For example, parasites in the control $A_{SCN}A_{food}$ group (orange) experienced hosts with synchronized SCN -and feeding/fasting-driven rhythms and sequestered at night during the feeding window. Whereas parasites in the $MA_{SCN}A_{food}$ group (purple) experienced hosts in which the phase-relationship between feeding-fasting and SCN–driven rhythms were disrupted, and parasites sequestered in alignment with the feeding window but misaligned to SCN-driven rhythms. We sampled blood and organs after 6 hours (red arrow) to estimate sequestration rates and sampled blood after 12-14 hours (blue arrow) to quantify parasites in the next IDC to estimate how well parasites in the different treatment groups replicated as a proxy for fitness. "ZT" refers to Zeitgeber time, for which ZT0 indicates lights on and ZT12-24 occurs during the dark phase.

Group 1 (orange in Figure 3.2, n=15, 5 females, 10 males) represent 'control conditions' in which host rhythms were unperturbed (standard photoschedule with lights on, ZT0, at 22:00GMT and off, ZT12, at 10:00GMT and *ad libitum* access to food, which mice predominantly eat during the active, i.e. dark phase). Under these conditions, host feeding-fasting and SCN-driven rhythms are synchronized with each other (i.e. follow their natural phase relationship). Trophozoites, harvested at ZT13 from donor mice, were introduced at ZT13 causing parasite rhythms to be aligned to both SCN-driven and feeding-fasting rhythms ("$A_{SCN}A_{food}$").

Group 2 (red in Figure 3.2, n=15, 5 females, 10 males) mice experienced the standard photoschedule (lights on 22:00-10:00GMT) and had food available during the light phase (ZT0-12) only. These conditions invert (disrupt) the natural phase relationship between feeding-fasting and SCN-driven rhythms. Trophozoites were introduced at ZT13, thus generating infections in which the IDC rhythm is aligned to SCN-driven rhythms, but 12 hours misaligned to feeding-fasting rhythms ("$A_{SCN}MA_{food}$").

Group 3 (purple in Figure 3.2, n=15, 5 females 10 males) mice had their photoschedule reversed (lights on 10:00- 22:00GMT) and food availability was restricted to the light phase (ZT0-12), thus inverting the natural phase relationship between feeding-fasting and SCN-driven rhythms. Trophozoites, were introduced at ZT1, generating infections in which the IDC rhythm is 12 hours misaligned to SCN-driven rhythms, but aligned to feeding-fasting rhythms ("$MA_{SCN}A_{food}$").

Group 4 (green in Figure 3.2, n=15, 5 females 10 males) mice had their photoschedule reversed (lights on 10:00-22:00 GMT) and experienced constant ad libitum access to food, ensuring feeding-fasting and SCN-driven rhythms are in sync with each other. Trophozoites were introduced at ZT 1, thus generating infections in which the IDC rhythm is misaligned to both SCN-driven and feeding-fasting rhythms ("$MA_{SCN}MA_{food}$").

We assume that introduced parasites continue their IDC at the same rate and so, are forced to sequester during the schizont stages regardless of the phase (timing) of the host's feeding-fasting and photoschedule driven rhythms. Thus, at 6 hours PI, five (female) mice per treatment group were euthanised to collect entire lungs, liver, spleen to assess abundance and 10 uL blood samples were collected to quantify parasite densities. Because the tissue processing required to directly assay parasites attached to endothelial cells (i.e. true sequestration) cannot be carried out in a high-throughput manner in a large-scale experiment, we use the abundance of parasites that accumulated within each organ of interest, as a proxy for sequestration. We estimated parasite biomass in the blood again during the subsequent IDC to assess the productivity of the introduced IDC cohort by euthanising the remaining ten (male) mice per treatment group at 12-14 hours PI and comparing the densities of circulating parasites in these mice to those in blood of mice sampled at 6 HPI.

Predictions

Comparing densities of parasites accumulated within each organ, between treatment groups, allows us to independently estimate whether sequestration opportunities are affected by SCN-driven and feeding-fasting rhythms, and by disruption of the alignment between host rhythms. For example, if only SCN-driven rhythms determine sequestration patterns within an organ, then the $A_{SCN}A_{food}$ and $A_{SCN}MA_{food}$ groups will exhibit similar patterns of abundance, which differ from the $MA_{SCN}A_{food}$ and $MA_{SCN}MA_{food}$ groups (which are similar to each other). In contrast, if only feeding-fasting rhythms determine sequestration patterns within an organ, then the $A_{SCN}A_{food}$ and $MA_{SCN}A_{food}$ groups will exhibit similar patterns of abundance, which differ from the $A_{SCN}MA_{food}$ and $MA_{SCN}MA_{food}$ groups (which are similar to each other). If sequestration rates are higher in a host exhibiting normal compared to disrupted rhythms, then the

$A_{SCN}A_{food}$ and $MA_{SCN}MA_{food}$ groups will exhibit similar levels of abundance, which differ from the $A_{SCN}MA_{food}$ and $MA_{SCN}A_{food}$ groups (which are similar to each other).

Furthermore, we anticipated that the different niche characteristics provided by the different organs might influence patterns of abundance. Particularly, ICAM1 expression is not rhythmic in the lungs and so at this site, we expected host SCN-driven rhythms will not affects patterns of abundance (i.e. $A_{SCN}A_{food}$ and $MA_{SCN}A_{food}$ perform similarly, as do $A_{SCN}MA_{food}$ and $MA_{SCN}MA_{food}$). Given the liver's role in food digestion, any influence of feeding-fasting rhythms in patterns of abundance and subsequent replication might be most apparent at this site. Whereas patterns of abundance in the spleen are more likely than other sites to be governed by rhythms in immune factors. Immune rhythms can be influenced by both the SCN and feeding-fasting schedule (Poole, et al., 2022; Zheng, et al., 2020) and so, might manifest strongest as a difference between groups in which parasites are matched ($A_{SCN}A_{food}$) versus mismatched ($MA_{SCN}MA_{food}$) to all host rhythms.

Finally, we expected that if sequestration enhances fitness via facilitating replication, groups with the highest abundance in tissues would exhibit the greatest increase in blood density between the introduced parasites and subsequent IDC.

Quantification of parasite and host transcripts

Parasite densities were quantified by quantitative RT-PCR targeting 18S rRNA, for which multiple copies per parasite are present, thus achieving a good sensitivity of detection (Pichugin and Krzych, 2015). Samples were processed as described below.

Organ samples

Upon dissection, each organ was cooled on dry ice and subsequently stored individually at -80°C. Each frozen organ was homogenized in 5mL Trizol™ (Invitrogen) using a Qiagen tissue-lyser with stainless steel ball-bearings at 30bpm for 4 minutes total. RNA was extracted from 1.5 mL of each homogenate, and then phenol:chloroform extraction and DNase treatment were carried out (Qiagen #79254)

following Pichugin et al., (2015) without organ perfusion. RNA samples were subjected to isopropanol precipitation to remove contamination with solvents carried over from manual extraction, and eluted in water. 1 µg of RNA was used to produce cDNA (high-capacity cDNA reverse transcriptase kit, Thermo Fisher Scientific) following the manufacturer's protocol.

Expression of parasite 18S rRNA is quantified relative to expression of the host reference gene mouse β-actin (Pichugin and Krzych, 2015). This relative expression allows for the quantification of changes in expression between samples of different treatment groups, and is not affected by a lack of control samples of known parasite densities for each tissue type, or variation in β-actin RNA expression levels between treatment groups, for each tissue type (Treatment: χ^2 (58)= 64.48, p = 0.26; χ^2(60)= 60.00, p = 0.48; χ^2(57)= 56.85, p = 0.48 for liver, lungs and spleen respectively), nor by mouse sex (Tissue by Sex interaction: LRT χ^2(2) = 5.81, p= 0.06; Treatment by Sex interaction: LRTχ^2(3) = 3.87, p = 0.28). However, we confirm previous observations (Kouadjo et al., 2007; Yan et al., 2016) that absolute levels of RNA expression of β-actin differ between tissue types (Tissue χ^2 (2) = 88.67, p= <2e-16) which, in addition to variation in RNA extraction efficiencies, prevents direct comparison across organs.

Briefly, 25µL qPCR reactions were set up separately for 18S and β-actin following (Pichugin and Krzych, 2015) and using their *P. berghei* primers. Because *in silico* matching of the *P. berghei* primers to *P. chabaudi* showed small inconsistencies (shortening matching primer sequences by 1-2 nucleotides and a single AT-mismatch in the reverse primer), we confirmed compatibility with *P. chabaudi* genotype AS reference samples. Assay conditions were: forward and reverse primers at 320nM, 1x SYBR Green PCR Mastermix (Thermo Fisher Scientific) and 2µL 1:10 diluted cDNA. Primers were 18SFW: 5'-AAGCATTAAATAAAGCGAATACATCCTTAC-3' and 18SRV 5'-GGAGATTGGTTTTGACGTTTATGTG (Pichugin, originally from Bhanot et al 2003 MBP 126(2), 263-273) or β-actinFW: 5'-GGCTGTATTCCCCTCCATCG-3' and β-actinRV: 5'-CCAGTTGGTAACAATGCCATGT-3'. The amplification program was as follows: 95°C for 15 min; 45 cycles of: 95°C for 20 seconds, 60°C for 30 seconds and 72°C for 50 seconds. We quantified relative gene expression of 18S rRNA using the

ddCt Pfaffl method (Pfaffl, 2001), to account for differences in PCR efficiencies between the target and housekeeping genes used.

Blood samples

Ten µL blood samples were stored in 20µL RNAlater® at -70°C. RNA from all blood samples was extracted using the MagMAX™-96 Total RNA Isolation Kit (Thermo Fisher Scientific), and DNAse treated, both following Schneider et al., 2019 (dx.doi.org/10.17504/protocols.io.88fhztn). To prevent downstream problems with inhibitors, samples were diluted 5-fold before producing cDNA (high-capacity cDNA reverse transcriptase kit, Thermo Fisher Scientific) following the manufacturer's protocol. Parasite densities per µL blood were quantified using the 18S rRNA assay described above, although quantification was based on a standard curve generated by measuring RNA expression in a 1:10 serial dilution series of a reference sample for which parasite densities were quantified by microscopy. This assay achieves a lower limit of detection of 6.62 parasites/µL blood, and all samples with <6.62 parasites/ µL blood were considered to be negative.

Data analysis

All analyses were conducted using R 4.0.2 (R Core Team, 2020). Initial analyses compared the four treatment groups by testing the effect of alignment within each host rhythm (i.e. feeding-fasting: aligned or misaligned, and SCN-driven: aligned or misaligned), and their interaction on the estimates of parasite abundance. Following this, post hoc tests were carried out to compare between treatments and investigate whether estimates of parasite abundance were influenced by (i) how many host rhythms the IDC was in alignment with (either both SCN-driven and feeding-fasting rhythms, one of them, or neither); and (ii) whether the phase relationship between host SCN-driven and feeding-fasting rhythms mattered (i.e. whether or not the natural phase relationship between host rhythms was disrupted). Specifically, we compared parasite densities from blood samples using generalized linear models with a negative binomial error distribution to account for overdispersion (R package glm.nb {MASS}), and model estimates (^{e}log parasite densities) are presented after back-transformation. Models were minimised using likelihood ratio test and results are presented as Chi-square values with associated degrees of freedom and p values. Kruskal-Wallis tests

were used to compare host b actin reference and the median counts (R package {stats}). For the organ data, relative gene expression was compared between treatments, using beta regression to take heteroscedasticity into account (tested by Breusch-Pagan tests, R package bptest {betareg}), minimising models via likelihood ratio tests (LRT) (R package lrtest {lmtest}). Wald statistics were used for constructing confidence intervals and p-values (R package waldtest {lmtest}). Finally, pairwise treatment contrasts were adjusted for multiple comparisons using the R function 'contrast' and estimated marginal means (estimates from predicted model values). Back transformed fold differences with a CI are presented for ease of interpretation, with z-ratios and p-values (R package {multcomp}{emmeans}).

Results

Patency, IDC progression and meeting experimental design assumptions

At 6 hours post infection (HPI), parasites were detectable in the blood in all treatment groups and densities were similar across treatments, with a predicted mean of 534 (95% CI 364 -785) parasites/µL across all groups (Treatment, χ^2 (3) = 2.40, p= 0.49; Figure 3.3). Furthermore, parasite densities were 2.14 fold (CI 1.43 – 3.44) higher at 12-14 HPI (estimate = 1,144, SE = 139.1, CI 872 -1,501) than at 6 HPI (estimate = 534, SE = 91.9, CI 364 - 785), validating our assumption that inoculated parasites underwent schizogony and re-invaded RBC between sampling points (z.ratio = -3.69, p < 0.001). In addition, we found no support for differences between sexes which allows comparisons between groups (Treatment by Sex interaction: LRχ^2(3) = 3.87, p = 0.28). Also, we confirm weights of mice under TRF (Timed restricted feeding) were not impacted by treatments disrupting natural feeding time (ANOVA repeated measurements, $F_{3,57}$= 1.369, p = 0.26). Thus, validating further comparisons between treatment groups.

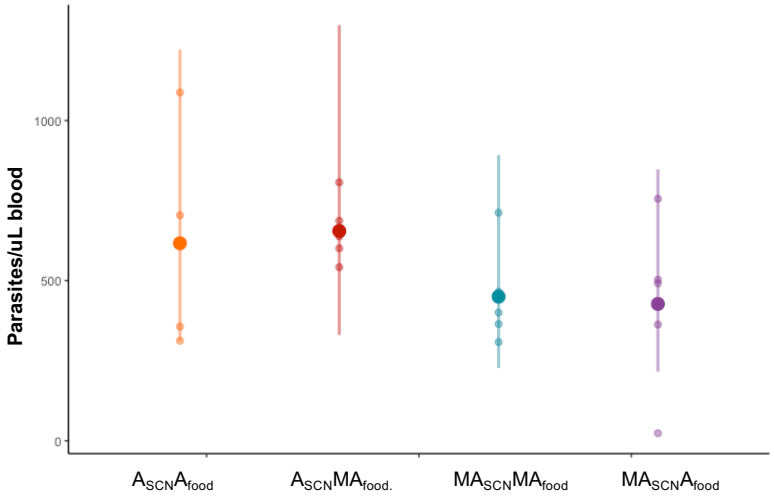


Figure 3.3. Parasite densities in the peripheral blood at 6 hours post infection. Predicted mean and 95 CI% are displayed with data points for individual mice plotted as open symbols. Colours represent the different treatment groups, which are coded as A = aligned or MA = misaligned to each host rhythm, with the host rhythms denoted in subscript ($_{SCN}$ for SCN-driven rhythms and $_{food}$ for feeding-fasting rhythms). Sample size n = 5 per treatment group.

Patterns of abundance: Liver

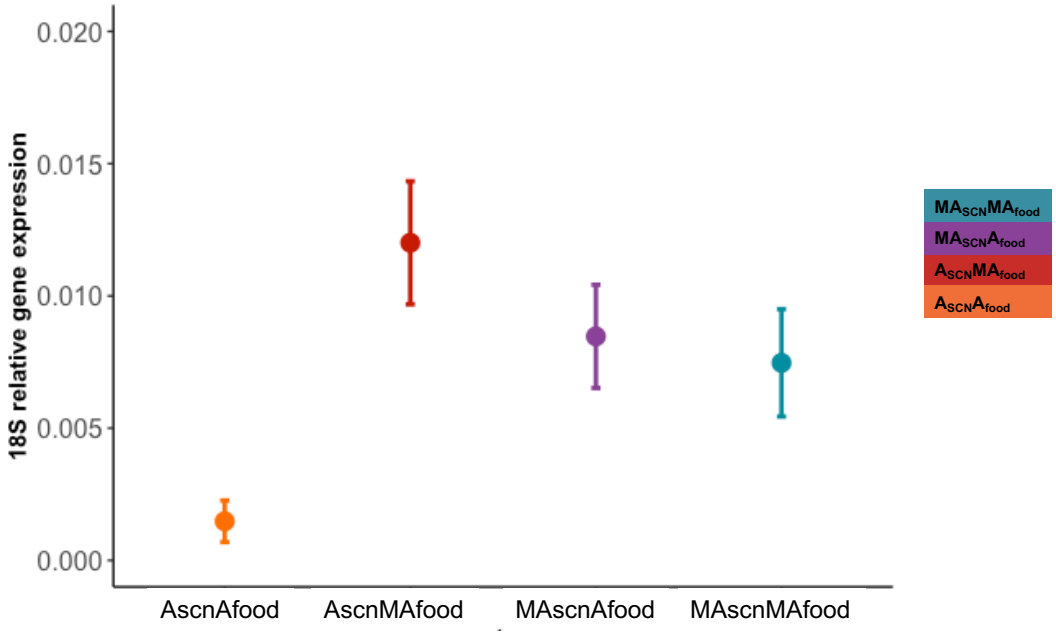


Figure 3.4. Relative 18S parasite expression according to the four treatment groups 6 hours post infection. Plots display predicted means and 95%CI, n=5.

Abundance (approximated by 18S relative gene expression) in the liver varied across treatments (Figure 3.4, LRT: $\chi^2(3) = 43.581$, p=<.0001). To further explore which host rhythms alignment (SCN or Food driven) altered accumulation we use decided to use interaction plots. Specifically, there was an interaction between whether sequestering

parasites were aligned or misaligned to each host rhythm SCN and food driven (LRT: $\chi^2(1) = 27.89$, $p < 0.001$, Figure 3.5a). Post hoc analysis revealed that parasite densities in the liver were similar (differing only 1.13 fold (CI 0.82 - 1.60) (z.ratio = 0.89, p = 0.37) in both treatment groups experiencing IDC misalignment to SCN-driven rhythms (MA_{SCN}) irrespective of alignment to feeding-fasting rhythms, and at a level intermediate to the other groups. The lowest abundance occurred in the control group which was aligned to both SCN-driven and feeding-fasting rhythms ($A_{SCN}A_{food}$ is 6.28 fold (CI 11.11 - 4.16) lower than the other 3 groups (z.ratio = -2.92, p = 0.003). Most parasites accumulated when aligned to SCN-driven and misaligned to feeding-fasting rhythms ($A_{SCN}MA_{food}$ is 1.51 fold (CI 1.15 - 2.01) higher than the MA_{SCN} groups (z.ratio=6.90, p = <.0001).

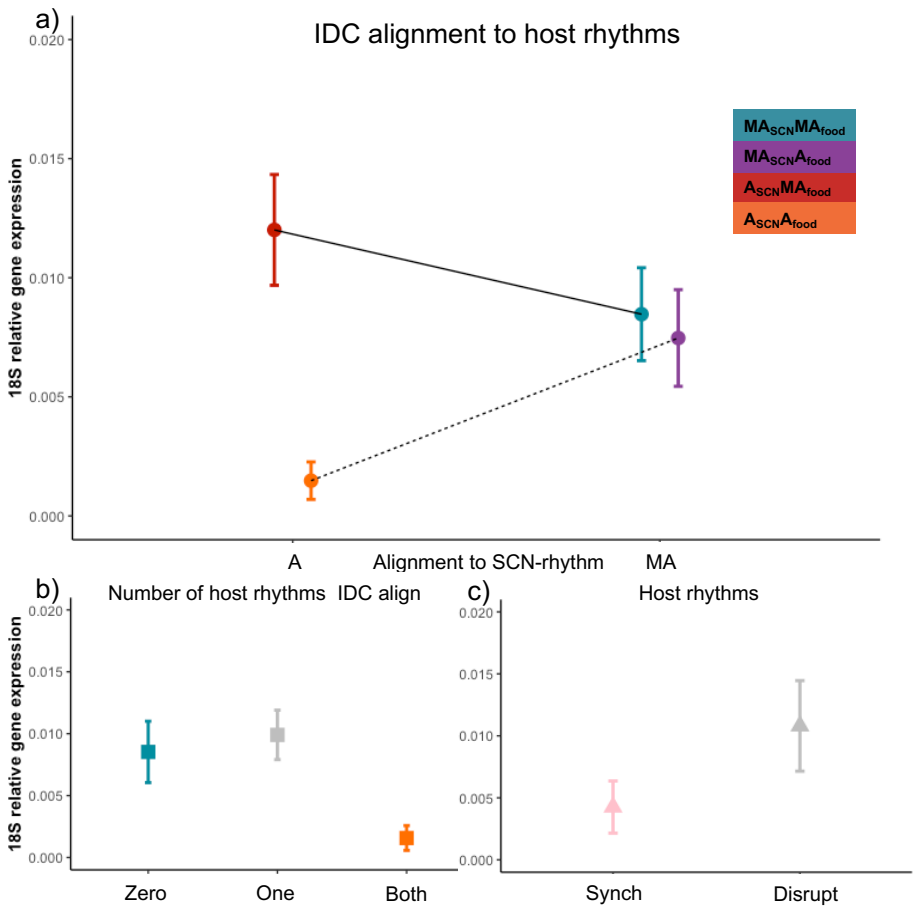


Figure 3.5. Parasite abundance in the liver at 6 hours post infection. Plots illustrate how parasite abundance (approximated by 18S relative gene expression) changes with respect to (a) IDC alignment to host rhythms, with all 4 groups plotted as an interaction plot with solid line connecting MA_{food}, and dotted line connecting A_{food} groups; (b) IDC alignment to neither (SCN or feeding/fasting; A_{ZERO}=$MA_{SCN}MA_{FOOD}$, teal), one rhythm (A_{ONE}=$A_{SCN}MA_{FOOD}$ and $MA_{SCN}A_{FOOD}$ combined, grey), or both host rhythms (A_{BOTH}=$A_{SCN}A_{FOOD}$, orange); and (c) Whether the host experienced synchronized or

disrupted rhythms, with post-hoc groupings “synch” combining $A_{SCN}A_{FOOD}$ and $MA_{SCN}MA_{FOOD}$ (pink) and “disrupt” combining $A_{SCN}MA_{FOOD}$ and $MA_{SCN}A_{FOOD}$ (grey). Plots display predicted means and 95%CI. n= 5 per treatment group.

We then conducted further post hoc analyses by combining treatment groups in different ways to investigate whether parasite abundance was affected by: (i) how many (zero, one, or both) host rhythms the IDC was aligned with, and (ii) disruption of the natural phase relationship between the hosts’ SCN-driven and feeding-fasting rhythms. Parasite abundance varied depending on how many host rhythms the IDC was aligned with (LRT $\chi^2(2)= 33.56$, $p < 0.001$, Figure 3.5b). Specifically, abundance was similar for groups in which parasites were aligned with one or neither host rhythms ($A_{ONE}/A_{ZERO,}$ z.ratio= 1.04, p = 0.891), and in keeping with the above, abundance was 6.29 fold (CI 10.20 – 4.41) lower in the control group in which parasites were aligned to both rhythms ($A_{ZERO\&ONE}/ A_{BOTH}$ z. ratio = -9.84, p = <.0001). This suggests that misalignment of the IDC with either host rhythm increases accumulation of parasites in the liver. Finally, comparing infections according to whether or not the host’s feeding-fasting and SCN-driven rhythms were synchronized with each other reveals parasite abundance was 2.56 fold (C 1.35 - 12.98) higher when the host’s rhythms are disrupted (LRT $\chi^2(1) = 10.43$, $p = 0.001$; z.ratio = 3.62, p <0.001; Figure 3.5c).

Patterns of abundance: Lungs

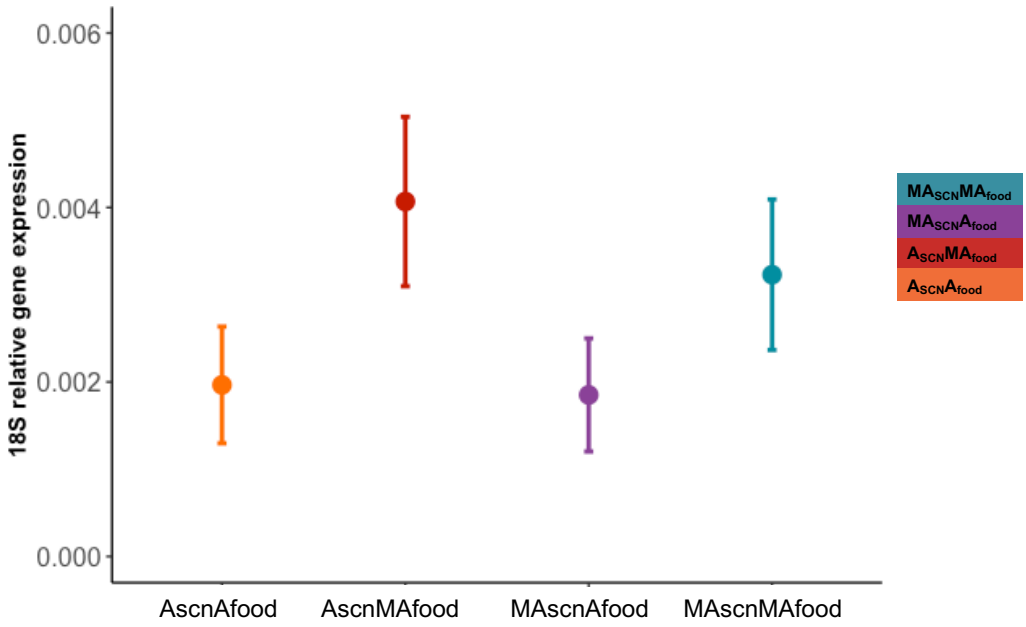


Figure 3.6 Relative 18S parasite expression according to the four treatment groups. Plots display predicted means and 95%CI, n=5.

Abundance (approximated by 18S relative gene expression) in the lungs varied across treatments (Figure 3.6, LRT: $\chi^2(3) = 18.069$, p=0.0004). Moreover, accumulation in the lungs, as for the liver, varied across treatments with an interaction between whether sequestering parasites were aligned or misaligned to each host rhythm (LRT, $\chi^2(1) = 17.17$, p <0.001; Figure 3.7a). Specifically, when parasites were aligned to feeding-fasting rhythms (A_{food}), abundance was 1.61 fold (CI 2.56 - 1.11) lower when parasites were also aligned to SCN-driven rhythms ($A_{SCN}A_{food}$) compared if misaligned to SCN-driven rhythms ($MA_{SCN}A_{food}$) (z.ratio= 2.90, p = 0.022). Similarly, for parasites misaligned to feeding-fasting rhythms, abundance was 2.22 fold (CI 3.44 - 1.51) lower when parasites were also misaligned to SCN-driven rhythm ($MA_{SCN}MA_{food}$) (z.ratio= -4.76, p = <.0001).

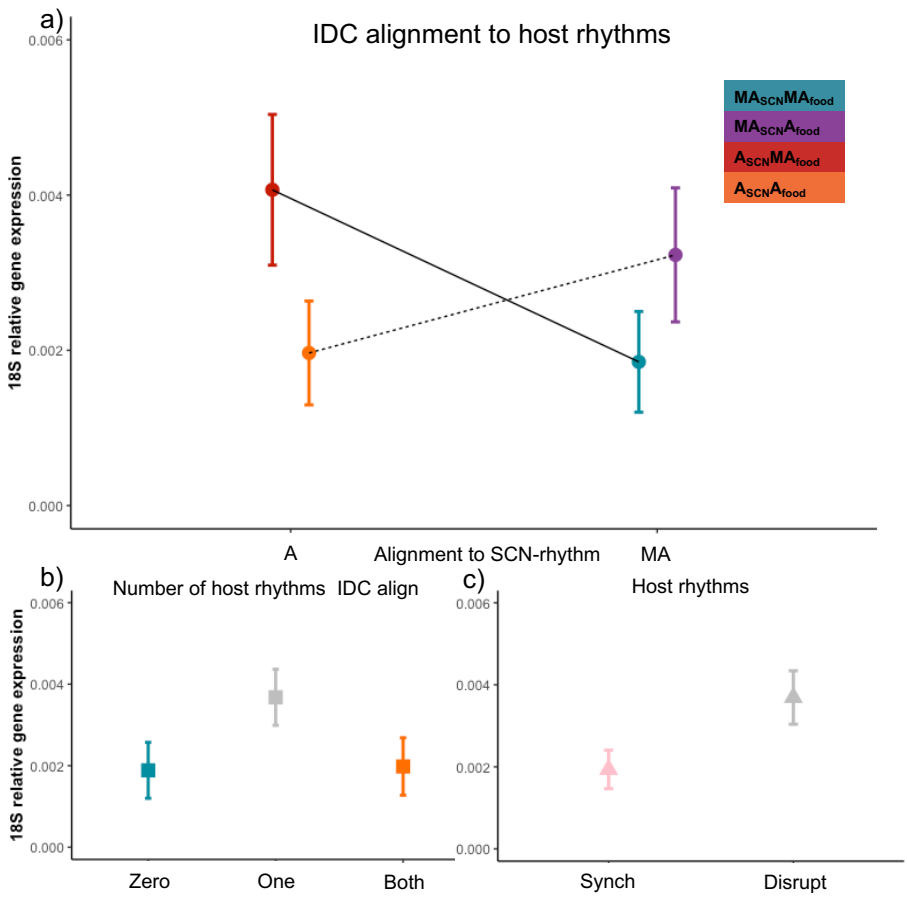


Figure 3.7. Parasite abundance in the lungs at 6 hours post infection. Plots illustrate how parasite abundance (approximated by 18S relative gene expression) changes with respect to (a) IDC alignment to host rhythms, with all 4 groups plotted as an interaction plot with a solid line connecting MA_{food}, and a dotted line connecting A_{food} groups; (b) IDC alignment to neither (SCN or feeding/fasting; A_{ZERO}=$MA_{SCN}MA_{FOOD}$, teal), one rhythm (A_{ONE}=$A_{SCN}MA_{FOOD}$ and $MA_{SCN}A_{FOOD}$ combined, grey), or both host rhythms (A_{BOTH}=$A_{SCN}A_{FOOD}$, orange); and (c) Whether the host experienced synchronized or disrupted rhythms, with post-hoc groupings "synch" combining $A_{SCN}A_{FOOD}$ and $MA_{SCN}MA_{FOOD}$ (pink) and "disrupt" combining $A_{SCN}MA_{FOOD}$ and $MA_{SCN}A_{FOOD}$ (grey). Plots display predicted means and 95%CI. Sample size n= 5 per treatment group.

In line with the above, post hoc tests shows that parasite abundance in the lungs was affected by how many host rhythms the IDC was aligned to (LRT $\chi^2(2) = 5.65$, $p = 0.0004$; Figure 3.7b). Specifically, abundance did not differ between parasites aligned to zero or both host rhythms (A_{BOTH}/A_{ZERO}, z.ratio = 0.23, p = 1.000), but was 1.86 fold (CI 1.61 - 2.19) higher when the IDC was misaligned to one host rhythm (A_{BOTH}/A_{ONE}, z.ratio = -4.16, p = 0.0001; Figure 3.7b). Finally, comparing infections according to whether the host's feeding-fasting and SCN-driven rhythms were synchronized or not reveals abundance is 1.90 fold (CI 1.49 - 2.51; z.ratio=4.95, p = <.0001) in hosts whose rhythms were out of synch (LRT $\chi^2(1) = 15.60$, $p < 0.001$) (Figure 3.7c).

Patterns of abundance: Spleen

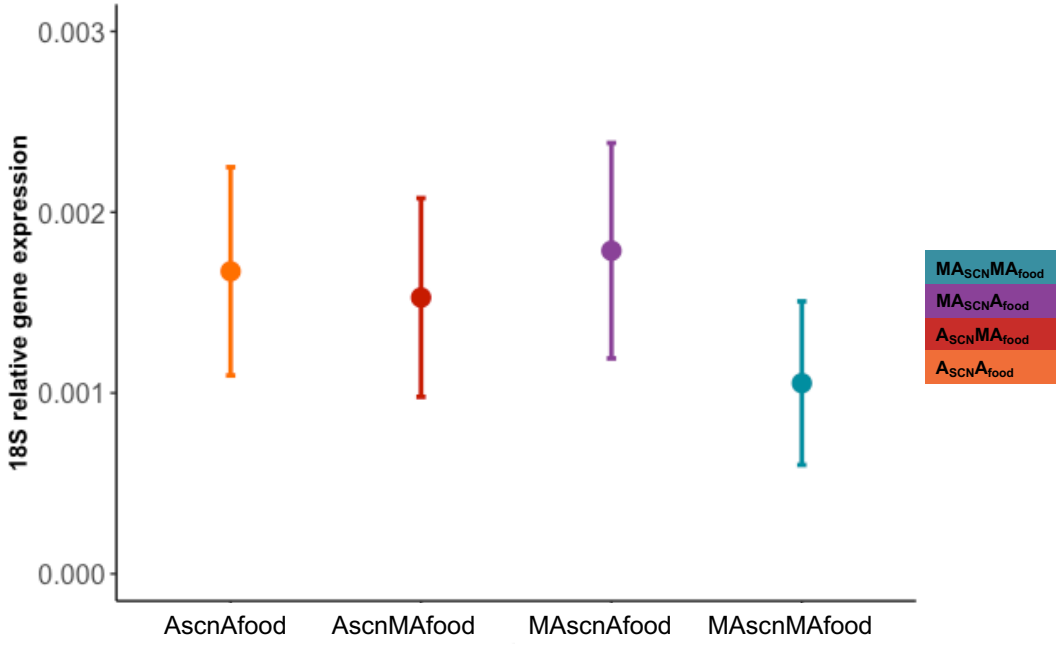


Figure 3.8. Relative 18S parasite expression according to the four treatment groups. Plots display predicted means and 95%CI, n=5.

Abundance (approximated by 18S relative gene expression) was quite low in the spleen (mean estimate = 0.001, SE= 0.00022). In the spleen no differences were detected across treatments (Figure 3.8, LRT: $\chi^2(3) = 6.3683$, p=0.095). Unlike in the liver and lungs, abundance in the spleen (Figure 3.9a), was not influenced by the interaction between alignment and host rhythms (LRT$\chi^2(1) = 6.574$, p= 0.087), nor the relationship to feeding-fasting (LRT$\chi^2(1) = 1.83$, p= 0.176) or SCN driven rhythms (LRT$\chi^2(1) = 0.904$, p= 0.3416). This is supported by the post hoc groupings revealing no influence of how many host rhythms the IDC was aligned to (LRT $\chi^2(2) = 3.75$, p = 0.15, Figure 3.9b), or whether the host's own rhythms were disrupted (LRT $\chi^2(1) = 3.62$, p = 0.06, Figure 3.9c).

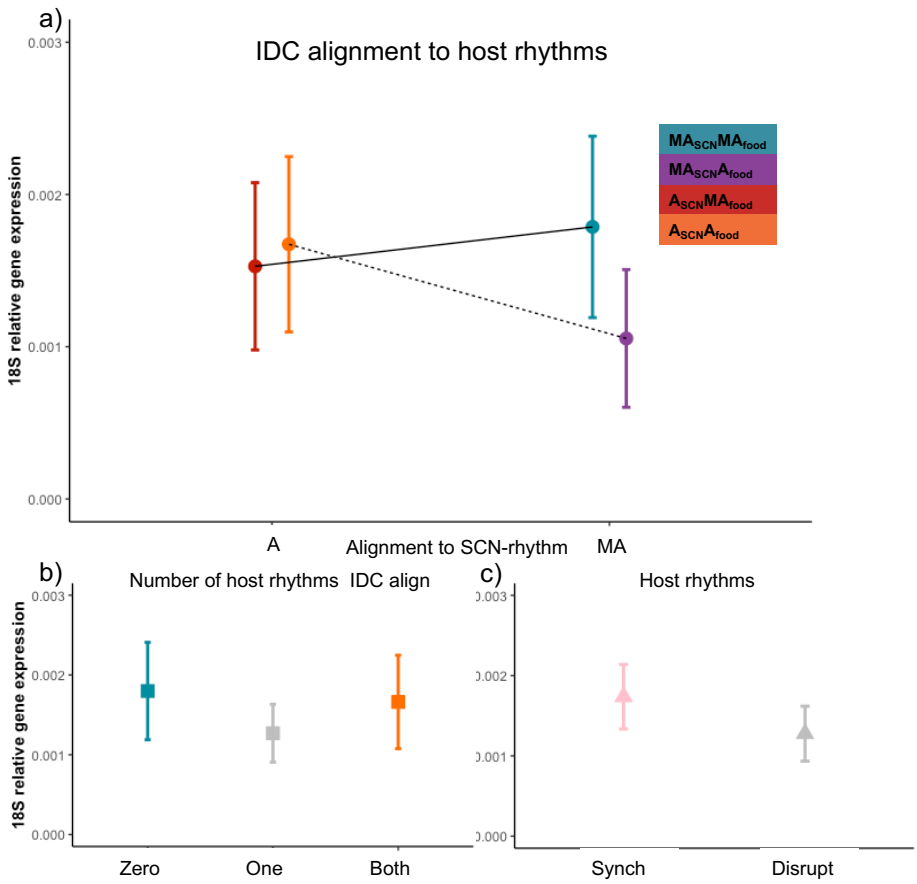


Figure 3.9. Parasite abundance in the spleen at 6 hours post infection. Plots illustrate how parasite abundance (approximated by 18S relative gene expression) changes with respect to (a) IDC alignment to host rhythms, with all 4 groups plotted as an interaction plot with solid line connecting MA_{food}, and dotted line connecting A_{food} groups; (b) IDC alignment to neither (SCN or feeding/fasting; A_{ZERO}=$MA_{SCN}MA_{FOOD}$, teal), one rhythm (A_{ONE}=$A_{SCN}MA_{FOOD}$ and $MA_{SCN}A_{FOOD}$ combined, grey), or both host rhythms (A_{BOTH}=$A_{SCN}A_{FOOD}$, orange); and (c) Whether the host experienced synchronized or disrupted rhythms, with post-hoc groupings "synch" combining $A_{SCN}A_{FOOD}$ and $MA_{SCN}MA_{FOOD}$ (pink) and "disrupt" combining $A_{SCN}MA_{FOOD}$ and $MA_{SCN}A_{FOOD}$ (grey). Plots display predicted means and 95%CI. Sample size n= 5 per treatment group.

Consequences: parasite densities

As reported in the "patency and IDC progression" section, parasite densities in the blood approximately doubled (increased by 2.14 fold (CI 1.43 – 3.44)) between 6 and 12-14 HPI, reflecting successful schizogony. However, neither the estimated parasite multiplication rate between sampling times (interaction of HPI by Treatment: LRT $\chi^2(3)$ = 1.28, p = 0.73) or parasite densities at 12-14HPI ($\chi^2(3)$ = 3.57, p = 0.31), were influenced by treatment (Figure 3.10a). Per treatment means are: $A_{scn}A_{food}$ =1580±380, $A_{scn}MA_{food}$ = 946±228, $MA_{scn}A_{food}$=1085±26, $MA_{scn}MA_{food}$ = 1042±251.

Furthermore, our post hoc tests revealed no influence of the number of host rhythms the IDC was aligned to ($\chi^2(2) = 3.52$, $p = 0.17$, Figure 3.10b), or whether the host's own rhythms were disrupted ($\chi^2(1) = 1.83$, $p = 0.18$, Figure 3.10c).

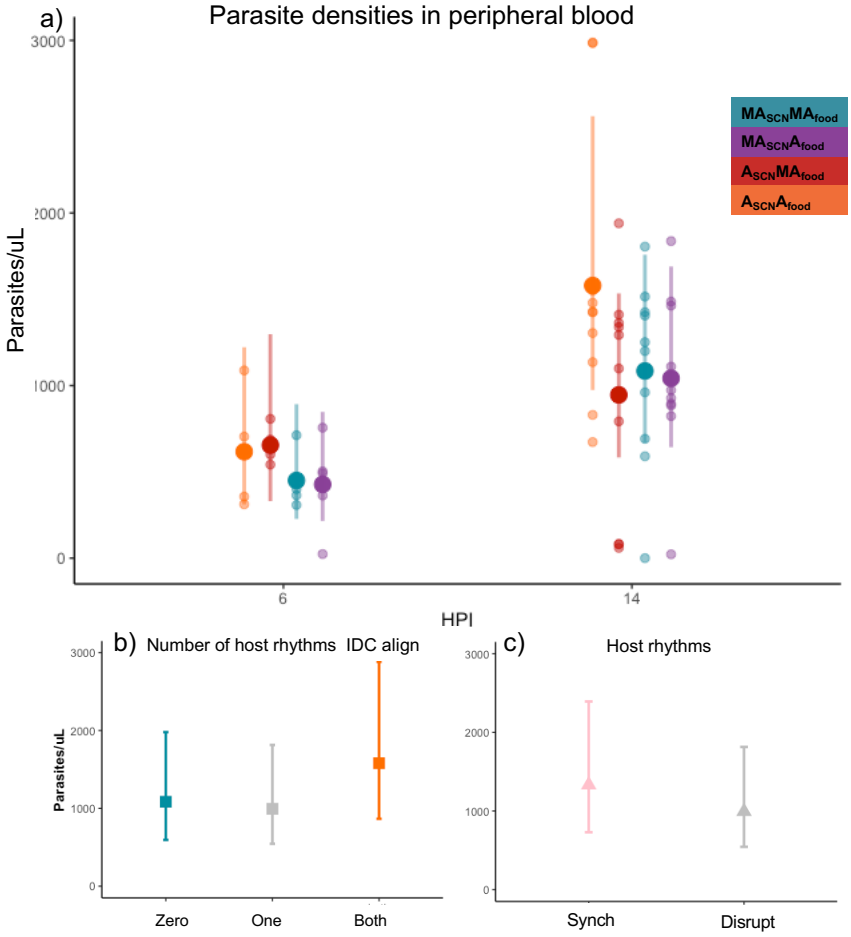


Figure 3.10. Densities of parasites in peripheral blood. (a) Big circles denote means and lines denote 95% CI from model predictions, with individual data points as small circles, at 6 hours (from Figure 3) and at 12-14 hours post infection; (b) IDC alignment to neither (SCN or feeding/fasting; A_{ZERO}=$MA_{SCN}MA_{FOOD}$, teal), one rhythm (A_{ONE}=$A_{SCN}MA_{FOOD}$ and $MA_{SCN}A_{FOOD}$ combined, grey), or both host rhythms (A_{BOTH}=$A_{SCN}A_{FOOD}$, orange); and (c) Whether the host experienced synchronized or disrupted rhythms, with post-hoc groupings "synch" combining $A_{SCN}A_{FOOD}$ and $MA_{SCN}MA_{FOOD}$ (pink) and "disrupt" combining $A_{SCN}MA_{FOOD}$ and $MA_{SCN}A_{FOOD}$ (grey). Plots display predicted means and 95%CI. Sample size n = 10 per treatment group.

Discussion

Here we opened the black box of the ecology of parasite sequestration in the context of host daily rhythms. We integrated knowledge of parasite rhythmicity in the IDC schedule, patterns of sequestration previously observed for asynchronously

replicating *Plasmodium spp.,* and host circadian rhythms, into an ecological framework. We first verified that the survival and replication of the introduced experimental parasites were not differentially affected by the perturbations used to create our treatment groups (Figures 3.3, 3.10). Having met this assumption of the experimental design we could compare parasite abundance across groups to probe for differences in sequestration rates. In contrast to our general, overall, expectations, we find that host rhythms have a subtle but variable role in shaping parasite sequestration across tissues. Overall, host rhythms affect parasite abundance in the liver and lungs but not the spleen (Figures 3.5a - 3.9a). In both the liver and lungs, infecting a host in which the phase relationship between SCN-driven and feeding-fasting rhythms is disrupted facilitates sequestration (Figures 3.5c, 3.7c), and most parasites sequester when misaligned to one of these host rhythms (Figures 3.5b, 3.7b). However, this variation in sequestration patterns does not impact on the replication of the focal IDC cohort (Figure 3.10).

Given the importance of feeding-fasting rhythms - which are largely driven by the liver - for IDC stages, we were surprised that sequestration in the liver was greater when the IDC schedule was misaligned to host feeding (Figure 3.5a). This suggests that rhythmicity in nutrients/resources acquired from the host's food are not a selective driver of sequestration activities. Put another way, sequestration is not scheduled to coincide with the appearance of resources in the liver microenvironment. Indeed, *P. chabaudi's* natural rhythm of sequestering in the feeding window may be a constraint which is compensated for by the benefits of acquiring rhythmic resources needed to produce asexual progeny and initiating the maturation of sexual transmission forms at a certain time of day (O'Donnell et al., 2022; Prior et al., 2021). Similarly, there is no consistent influence of SCN-driven rhythms on sequestration either. The highest level of sequestration was exhibited by parasites misaligned to feeding-fasting and aligned to SCN rhythms. Potentially, sequestering during the feeding window might expose parasites in the liver to higher oxidative stress than during the fasting window when metabolic rates are slower and explaining why sequestration in the fasting window is beneficial. This benefit might only produce a net gain for parasites when aligned to SCN rhythms because they are also leaving a potentially more oxidatively damaging in the blood. Testing these ideas requires future studies to characterise the

microenvironments in the liver and blood around the clock, and determine what constitutes dangers and opportunities for sequestering parasites.

In the lungs, our expectation that the timing of SCN-driven rhythms do not influence sequestration (because expression of main receptor parasites use to sequester does not oscillate, (He et al., 2018) is not well supported (Figure 3.7a). However, the finding that being misaligned to either the feeding-fasting or the SCN-driven host rhythm, but not both, increases sequestration in the lungs is intriguing. Being misaligned to feeding-fasting rhythms might benefit parasites sequestering in the lungs for the same reason as the liver; potentially avoiding sequestering in a more oxidative organ, whilst sequestration when out of synch with SCN-driven rhythms allows parasite to evade a dangerous environment in the blood. Such a scenario could involve rhythms in pulmonary innate immune responses which can be synchronised by SCN-driven systemic glucocorticoid signalling (Tognini et al., 2017). In this case, the pattern of the interaction (Figure 3.7a) could be explained by alignment with SCN rhythms resulting in exposure to more dangerous immune responses which erodes the benefit of being misaligned to the feeding window for the $A_{SCN}MA_{food}$ group compared to the $MA_{SCN}MA_{food}$ group. Conversely, the $MA_{SCN}A_{food}$ group experiences less dangerous immune responses but greater oxidative stress compared to $A_{SCN}MA_{food}$, explaining why these 2 groups exhibit similar levels of sequestration. However, if only two rhythms were involved in this manner, parasites misaligned to both feeding-fasting and SCN-driven rhythms ($MA_{SCN}MA_{food}$) should experience the most permissive environment, whereas those aligned to both ($A_{SCN}A_{food}$) should experience the harshest environment; which is not consistent with these groups exhibiting similar levels of sequestration. Thus, a third factor such as rhythmicity in lung microbiota might interact with immune and/or oxidative rhythms to shape sequestration. Whether microorganisms in the lungs exhibit their own daily rhythms in abundance or diversity appears to have been overlooked, but host circadian rhythms do affect some respiratory bacteria and virus (Edgar et al., 2016; Gibbs et al., 2014). For example, disrupting microbiota and their by-products that alter lung physiology (including inflammation), in mice faced with an endotoxin challenge alters the circadian lung transcriptome and metabolome (Murakami and Tognini, 2019). Thus, the activities of *Plasmodium* in the lungs could be affected by complex feedbacks between rhythmic host processes and their impact on respiratory microbiota. Further complexity may lie

in the potential for immune rhythms to be influenced by both the timing of SCN clocks and feeding, especially in the context of an acute infection. And even less is known about how these interactions are shaped by the activities of lung microbiota. Severe manifestations of parasite sequestration in the lungs in mice and humans both have been linked to changes in lung microbiota (Mukherjee et al., 2022). However, in the mouse model *P. berghei* altering the microbiota resulted in no changes in the biomass (sequestration rate) between days (Mukherjee et al., 2022). Future experiments could use gnotobiotic mice to test for roles of host rhythms in the absence of lung microbes.

A consistent finding across the liver and lungs is that sequestration is facilitated by disrupting the temporal alignment between the host's SCN-driven and feeding-fasting rhythms. Thus, an alternative to the time of day specific influences of rhythms on parasites sequestering in the liver or lungs (explored above) is that being misaligned to any, but only one, host rhythm is beneficial simply because it is unpinned by disrupting the host's rhythms. For example, perturbing temporal homeostasis may impair the host's ability to mount innate immune responses against sequestering parasites independently of the time of day. This could be more apparent in the lungs than the liver, because this site provides perhaps the best example of circadian regulation of immunity (Pariollaud et al., 2018; Nosal et al., 2020) and these results fit with Brugat et al., 2014 findings of accumulation varying between tissues across days possibly driven by host factors. In contrast, our study was unable to detect any treatment effect on the spleen accumulation which should have the highest accumulation at an early infection according to (Brugat et al., 2014). Nevertheless, within a day host tissue oscillations might be on slightly different phases therefore the peak for optimal accumulation might vary between tissues although driven by similar oscillation (Zhang et al., 2014). In addition to abundance being closer to the detection limit in the spleen, which might reduce power, 6 hours is unlikely to have been sufficient to generate splenic responses (i.e. clearance rate (Khoury et al., 2017) of naïve mice in any treatment groups). Future studies could address this by activating the spleen, via injecting hosts with killed parasites for example, prior to introducing the experimental parasites.

Another clear finding is that following schizogony, parasite densities in the blood did not vary between treatment groups (Figure 3.10). This suggests that parasites

proliferate equally well regardless of when and where they sequester, and that the seeming benefits of sequestering in a host with disrupted rhythms are too small to result in a significant impact on the replication rate. It is possible that the costs/benefits of sequestering in different organs at different times of day with respect to different host rhythms all cancel each other resulting in the same net replication. However, costs/benefits may be too small to detect in the first cycle of replication. Like the consequences of the IDC being misaligned to host rhythms (O'Donnell et al., 2011; O'Donnell, Mideo and Reece, 2013), the costs/benefits of sequestration rhythms might accrue as infections progress. This scenario is especially likely if immune evasion is a key selective driver of sequestration (Sherman, et al., 2003); such responses need time to develop and exert their (rhythmic) effects. Future work could repeat the experiment with pre-exposed mice (similar to (Gilks et al., 1990)) or by inducing an inflammatory phenotype (with, for example, LPS injection) before introducing experimental parasites. Imposing different time lags between immune perturbations and infections might also explain observations that adaptive immunity is not the driver of chronicity in *P. chabaudi* (Brugat et al., 2017), and that avoiding spleen clearance is not the only benefit for sequestration in *P. berghei* (Fonager et al., 2012). Furthermore, because we examined parasites at the beginning of infection when densities are very low, our design is unable to detect fitness consequences of sequestration that depend on parasite density.

The evolutionary ecology of sequestration as a "life history trait" is poorly understood. A key challenge for such studies – that require considerably more statistical power than reductionist studies of mechanism – is the lack of high throughput and quantitative assays for sequestration. It is not possible to perfuse tissues or to perform live imaging on a large number of hosts/organs/treatment groups in a timely manner. Thus, our approach is limited by using the abundance of parasites within tissues as a correlate of sequestration. We expect this will have added noise to our data and so, potentially obscured some differences between treatment groups. Though we expect that large differences between groups should have been detected. Furthermore, transmission stage (gametocyte) abundance is affected by host rhythms (Birget et al., 2017; Carter et al., 2013) but we did not assay gametocyte abundance within tissues. Due to the short time-scale in our experiment, gametocyte densities would simply reflect the levels of sexual commitment adopted by the experimental parasites when

they were still in the donor hosts, but perhaps gametocytes and asexuals are differentially affected by host rhythms in their sequestration locations and/or rhythms. Future work could include gametocyte assays as well as sampling locations they specifically home to, such as the bone marrow (Brugat et al., 2014; Nahrendorf et al., 2021). Recent work reveals that some *Plasmodium* spp. (including *P. berghei* and *P. vivax)*, undergo asexual replication in protective niches such as the bone marrow (De Niz et al., 2018; Venugopal et al., 2020) which opens up the possibility that host rhythms govern replication in these sequestration sites, as they do for IDC stages.

Conclusion

Parasite sequestration underpins the pathogenic outcome of malaria. The process of sequestration is complex, interacting with immune responses, host vascular regulation, and other characteristics of the microenvironment. Our study illustrates how to explore this complexity and suggests that disrupting within host rhythms can enhance sequestration. Such a phenomenon might occur in natural infections when hosts progress to the symptomatic stage and their rhythms become perturbed (Prior et al., 2019), adding to the severity of infections. Our findings also hint at higher levels of sequestration when the IDC is misaligned to some host rhythms; which we suggest might be due to rhythmicity in the risk of oxidative damage to parasites sequestering in the liver during the feeding phase when metabolic rates are high. If this hypothesis proves correct, then parasites following their natural IDC schedule are forced to sequester in a dangerous place at the most dangerous time of day, potentially revealing a constraint that interventions could exacerbate to reduce parasite fitness.

Chapter 4. Impacts of manipulating innate immunity on within host malaria parasite infection dynamics

Abstract

Decades of studies detail molecular and cellular mechanisms of host immune responses against malaria infection. Yet, many studies offer conflicting results so it remains challenging to quantify the functional impact of innate immune responses for controlling parasite burden and for host health outcomes. We conduct a broad meta-analysis to probe for consensus for the effect innate immune responses have on malaria parasite replication, focussing the early acute phase of three different species rodent malaria infections. Screening published studies that span four decades of research, we collate, curate, and statistically analyse parasite dynamics to identify and quantify consensus and identify sources of disagreement among studies. Additionally, we estimate whether host factors, experimental methodology, as well as the scale of perturbations, shape the impact of immune manipulations on parasite burden. First, we detected meta-analytic mean effect sizes (absolute Cohen's h) for the difference in parasite burden between control and immune-manipulated groups ranging between 0.149 and 0.233 across the three *Plasmodium* species. This range is considered a small effect size and translates to a parasitaemia difference of roughly 6-12% at the peak of infection. Second, we reveal that variation in the dynamics of *P. chabaudi and P. yoelii* infections are better explained by stochasticity due to small sample sizes than by the effects of experimental perturbations. Third, for *P. berghei,* the impacts of immune interventions are larger when younger, or female, mice are used as hosts, and greatest when effector molecules or networks are perturbed (resulting in an 18% difference in peak parasitaemia between control and treatment groups). Fourth, we found little evidence of publication bias suggesting our results are robust. Meta-analysis is a valuable tool for resolving whether innate components play critical roles in shaping parasite dynamics. Unfortunately, only 140 of the 1,488 relevant studies reported data in a manner appropriate for meta-analysis. We recommend the field standardises reporting of parasite dynamics to maximise the future value of data and maximise the ability to translate understanding from model systems to human malaria infections, manage immunopathology, and facilitating realism in mathematical models.

Introduction

Host immune responses play a pivotal role in shaping the fate of infectious agents and host health outcomes (Graham & Tate, 2017). Innate immune effectors can directly kill or block pathogen invasion, or kill the host cell they reside within (e.g. via ROS, complement, phagocytosis). For naïve hosts that have no previous exposure to malaria (*Plasmodium*) parasites, the innate arm of immunity is the primary response during the acute phase of malaria infection. Innate responses control parasite proliferation by targeting and removing infected red blood cells (RBCs) as well as short-lived extracellular parasites known as merozoites (Götz et al., 2017; Stevenson & Riley, 2004). By slowing parasite replication, innate defences are thought to allow sufficient time for the development of adaptive immunity. Indeed, in *P. falciparum* infections of humans, timely innate inflammatory responses are key for protection from severe disease (Artavanis-Tsakonas, Tongren, and Riley 2003). The initial phase of malaria infection is characterized by a proinflammatory environment with elevated concentrations of the cytokines Tumour necrosis factor (TNFα) and Interferon gamma (IFNγ) in the circulation (Götz et al. 2017). Failure to control inflammation can result in self-damage (i.e., immunopathology) and severe disease manifestation (Lamb et al., 2006). To minimise such damage, hosts activate disease tolerance mechanisms that alleviate inflammatory phenotypes (Nahrendorf et al., 2021b). Diversity in malaria infection outcomes, that range from subclinical to fatal, even within the same host-parasite combination, may be explained by individual variation in how innate immune responses mediate the balance between tolerance and resistance.

Establishing a causal pathway between specific immune mechanisms and clinically relevant malaria tolerance and / or resistance phenotypes – including *Plasmodium* proliferation and anaemia – has proved challenging. For example, *Plasmodium* sometimes elicits dysfunctional phenotypes of macrophages and dendritic cells (reviewed in:(Chua et al., 2013; Yap et al., 2019). Further complications arise because innate immune effectors may have multiple effects, for example, TNFα may not substantially alter parasite proliferation dynamics directly (but see (Sam & Stevenson, 1999), but is linked to immunopathology and disease manifestation (Hernandez-Valladares et al., 2006; Li et al., 1999; Mahittikorn et al., 2022). Redundancy in the innate immune system also makes it difficult to quantify the impacts of individual

components on parasite burden and infection severity. Consequently, it is challenging to find consensus on the extent to which innate immunity controls malaria parasite replication, how, and under what circumstances. To address this, we conduct a systematic and quantitative synthesis of the primary literature asking to what extent, and in what contexts, experimental manipulations of innate immunity impact the dynamics of parasite replication in rodent malaria infections.

Following the discovery of rodent malaria species in the 1940s, model systems have been developed to provide insight into all aspects of host-parasite interactions, including vast mechanistic knowledge of immune responses to malaria infection (Kirkman & Deitsch, 2020; Stephens et al., 2012a). Rodent malaria model systems offer a balanced compromise between manipulability and ecological realism when compared to infections of humans and *in vitro* systems. Specifically, while studying human infections offers the maximum clinical translational value, this is tempered by the difficulties of controlling for confounding factors in natural infections, and manipulations of the immune system not being possible due to ethical considerations (Chavatte & Snounou, 2021). At the opposite end of the spectrum, *in-vitro* model systems offer freedom of manipulation to probe molecular and cellular mechanisms in detail but lack realistic within-host ecological processes that regulate natural immune responses (Brown & Guler, 2020). Moreover, despite important differences to human malaria infections, the rodent malaria systems provide general insights into how host immunity controls acute malaria infections (Olatunde et al., 2022).

Many rodent malaria studies of innate immunity record an aspect of parasite proliferation (e.g., parasitaemia, the proportion of red blood cells infected with malaria parasites) following an experimental perturbation of the immune system. For example, approaches to study the impact of nitric oxide synthase 2 (NOS2, an enzyme involved in producing a reactive free radical), include infecting mice genetically lacking nitric oxide synthase 2 (e.g., Green et al. 1994) or treating mice with a NOS2-inhibitor drug (aminoguanidine) (e.g., van der Heyde et al. 2000) upon parasite inoculation. Via genetic and/or chemical interventions, such experiments offer insights into the impact of the manipulated immune component and its associated pathways on parasite burden. Immune-intervention experiments have been conducted in a variety of host genetic backgrounds and parasite species (i.e., *Plasmodium chabaudi, P. berghei*,

and *P. yoelii*) that offer diverse infection phenotypes. For example, *P. chabaudi* is a classic model of blood-stage *P. falciparum* human malaria while *P. berghei* causes a lethal infection that leads to cerebral malaria (Brian De Souza et al., 2010). As well as targeting different components of the innate immune system in alternative ways, experimental designs also vary in parasite injection routes and dose, and the age and sex of hosts. We take advantage of the flexibility of the rodent malaria models that have been deployed over several decades of studying the immunology of acute phase malaria infection. By collating, curating, and statistically analysing parasite dynamics from published studies that manipulate aspects of innate immunity, we take a meta-analytic to approach identify and quantify consensus and sources of disagreement among studies. Additionally, we apply meta-regression models to estimate whether host factors (e.g. age, sex), methodologies (including genetic manipulation, adoptive transfer and the injection of monoclonal antibodies or recombinant cytokines) as well as minor and major perturbations (e.g. loss of a single cytokine receptor versus deletion of the entire myeloid compartment), and statistical power of studies, shape the impact of immune pertubations on parasite burden (Harrer et al., 2021).

Meta-analyses quantitatively synthesise results from multiple studies using the standard effect size (absolute Cohen's h) to identify common results from studies whose data can be used to ask the same empirical question (Nakagawa et al. 2012; Gurevitch and Nakagawa 2015). This is a powerful approach because the measurements reported in each individual study are expected to have some degree of error and a meta-analysis accounts for this to derive a pooled estimate of the overall pattern across data sets, as well as reveal sources of disagreement among studies, or other interesting relationships that may come to light with multiple studies (Borenstein Michael, 2009). Our meta-analysis reveals a small average effect of experimental interventions of innate immunity, equivalent to a roughly 6 to 12% difference in parasitaemia of control and treatment groups at the peak of infection across species. Meta-regressions reveal that variation in the dynamics of *P. chabaudi and P. yoelii* infections are better explained by stochasticity due to small sample sizes than by the effects of experimental perturbations. However, for *P. berghei,* the impacts of immune interventions are larger when younger, or female, mice are used as hosts, and greatest when effector molecules or networks involved in innate immunity are

perturbed (an 18% difference in peak parasitaemia between control and treatment groups).

Methods

Our meta-analysis began with a systematic literature search. We then assessed the suitability of individual articles for inclusion before data were extracted from suitable studies and effect sizes calculated. The first phase of analysis was to calculate the overall effect size (i.e., meta-analytic mean) for each parasite species using a random-effects meta-analysis and to estimate heterogeneity (i.e., variability among studies that cannot be attributed to differences in sample sizes and may be explained by moderators relating to host factors or aspects of experimental design). The second phase was applied to *P. berghei*, which was the only species for which we detected non-zero heterogeneity, we explored the effect of biological and methodological moderators (i.e., potential explanatory variables of heterogeneity) using a series of univariate meta-regressions. Finally, we assessed publication bias (i.e. whether the probability of publication depends on the results of each study) graphically and using statistical tests.

Literature search, eligibility screening and data extraction

Our dataset consists of published articles reporting experimental manipulations of innate immune components in mice infected with rodent malaria parasites. We identified relevant articles with systematic searches of keywords as well as forward and backward citation searches. For our keyword search, we used the following keywords – innate immunity AND (*Plasmodium* OR rodent malaria) NOT mosquito* NOT human – in four databases (Pubmed, Scopus, Jstor, WoS) in January 2020. We filtered for research articles only and applied no restriction in the year of publication. We screened each article for inclusion in the dataset by first removing duplicates among searches, and then assessed the title and abstract of each article to check the experimental design and results consider a relevant topic. We then assessed the full content (where the full article was available) and retained articles (Additional file 1) that met the set of criteria (stated in Table 4S1) to include their data in the dataset for analysis. We also selected the ten oldest and newest articles that met the criteria for inclusion from the keyword search and conducted forward and

backward citation searches to identify and screen relevant articles that are cited by and citing each focal article. We followed the PRISMA ECOEVO 2021 guidelines for reporting systematic reviews and meta-analysis (Figure 4.1) (O'Dea et al., 2021). Overall, from an initial pool of 1488 articles (652 keyword, 457 forward, and 379 backward searchers), 84 articles - 47, 20 and 17 from the keyword, backward, and forwards searches, respectively – met the criteria to included in the dataset for analysis.

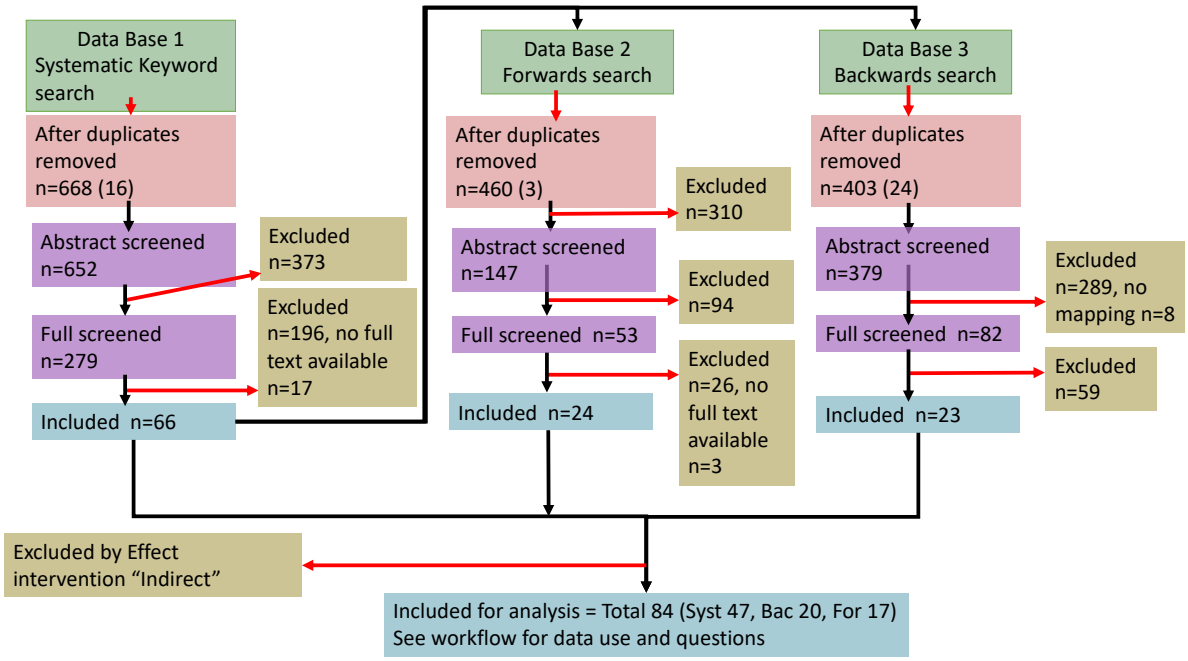


Figure 4.1. PRISMA flowchart of the literature search and data extraction. Our complete dataset included 84 articles for analysis of innate immune interventions. Box colours indicate literature search (green), screened (pink, purple), included (blue), excluded studies (yellow), and duplicates that were removed (red arrows, number removed inside parenthesis). The following abbreviations refer to: n = number of articles, Syst = Keyword search, Bac = Backwards, For = Forwards.

Data extraction

Our study focuses on analysing patterns of within-host parasite replication under different immune perturbations of *in-vivo* rodent malaria infections. The best metric for parasite burden is density (i.e. concentration in the blood) but this is rarely reported so we extracted data as parasitaemia (i.e., the proportion, or percentage, of infected red blood cells, iRBC), and we required studies to report multiple temporally spaced samples for both control and treatment groups. We focus on the acute phase of infection to minimise the influence of other host factors (e.g., RBC

depletion/replenishment) that can confound the impacts of immune responses on patterns of parasitaemia (which is affected by the change in the number of RBCs). Thus, we include data up to the peak of infection for *P. chabaudi* and non-lethal *P. yoelii*, which we define as the day post infection that either the control or treatment group reaches its maximum reported parasitaemia value. We include all data for *P. berghei* and lethal *P. yoelii* infections because these infections are usually terminated before reaching their peak. We analysed the four parasite groups (i.e., *P. chabaudi*, *P. berghei*, non-lethal and lethal *P. yoelii* infections) separately as parasite virulence and hence the pace of infection dynamics differ markedly among them. When articles included multiple experiments, each suitable experiment was included individually. All data points from figures were extracted using Datathief III v1.7(2015). AHM conducted all data extraction, and TK validated data points. The classification of the immune covariates was determined by AHM and PS.

Effect size – Cohen's h

We used the Cohen's h, which is commonly used to quantify the difference between proportions, as the standardized effect size (Cohen, 1977). We calculated Cohen's h for each study as the arcsine-transformation of the difference in parasitaemia in the control group compared to that in the treatment group (Rosenthal, 1994). Our study includes experimental manipulations that are expected to both augment (e.g. injecting rIFNg (Clark et al., 1987)) and hinder (e.g. cell depletion of phagocytic cells (Terkawi et al. 2016)) immune activities, which could result in retarding or facilitating parasite replication, depending on the approach used in each study (Atallah et al., 2020). Thus, if the raw effects were simply combined across experiments, perturbations with a negative impact on parasite burden may be cancelled out by those with a positive impact. To overcome this, we use absolute effect size (i.e. the non directional of the raw effect) in all analyses. Put another way, our estimates of Cohen's h do not indicate that control groups have a higher parasitaemia than treatment groups (or vice versa), just the magnitude of the difference between groups. For ease of interpretation, we also provide an estimate of the overall effect in terms of the magnitude of the parasitaemia difference between control and treatment groups at their peaks by back-transforming the meta-analytic mean conditional upon the control parasitaemia.

Analysis

Meta-analysis

We used mixed-effects meta-analytical models and all analyses were conducted in R 4.0.2 (R Core Team, 2020). We used the rma.mv function, from the metafor package (Viechtbauer, 2021), applying the cluster robust var-cov sandwich-type estimator to adjust for small samples. To account for repeated measurements due to multiple effect sizes collected from the same subjects (infections) over time, we included experiment identity as a random effect. We did not control for non-independence arising from rare experiments sharing the same control group. We examined possible publication bias in our meta-analysis using funnel plots, regression tests and rank tests of the raw effect size. We report heterogeneity among studies as the I^2 statistic (i.e., as the percentage of variance between effect sizes that cannot be attributed to sampling error due to small sample sizes) (Higgins et al., 2003).

Meta-regression

Only the *P. berghei* dataset revealed residual heterogeneity (i.e., variation associated with immune perturbations that cannot be explained by small sample sizes) which allowed us to conduct meta-regression analyses to explore sources of this variation. Moderator information was only available for subsets of data so we carried out a series of three univariate meta-regression analyses to examine the moderating effects of host, immunological, and experimental design factors on the impacts of immune interventions, as follows:

- Host factors
    - Age (centred median age reported in weeks; 5.5 to 11 weeks)
    - Sex (females, males, or unknown/reported in studies as mixed)
- Immunological factors
    - Signalling position in network (input or output),
    - Expected systemic phenotype (inflammatory, regulatory, or trafficking),
    - Cell lineage (lymphoid, myeloid or both)
    - Cytokines/chemokine or their receptors
- Experimental design factors
    - Sampling regime (day post infection)
    - Injection route for inoculation of parasites (intraperitoneal or intravenous)
    - Infection dose (number of parasitised RBC used to initiate infection)

- Manipulation type (drug, genetic modification (GM), surgery, cell transfer, or mixed (any combination)

Thus, our first analysis examined whether the age and / or sex of host influenced how much immune interventions alter parasitaemia, and compared their impacts. We were unable to assess the effect of host strain because the literature contains too many ambiguous categories within strains to derive a meaningful generalisation. Likewise, our second model compared the impacts of four different classifications of innate immune components, and our third analysis compared four different elements of experimental methods.

We conducted model comparisons using the Akaike Information Criteria corrected for small sample size (AICc) and log-likelihood ratio tests (LRT), and tested for differences between levels of a given moderator using linear hypothesis tests (Viechtbauer, 2021). Univariate models proved the most appropriate approach due to missing data and/or small sample sizes (Table 4S2) whilst noting that some meta-regressions involve unbalanced samples which may confound the results (Harrer et al., 2021). Meta-regression analysis was performed when the dataset contained more than ten data points (see Table 4S2 for detailed sample sizes across moderator levels).

Results

Overall effect size and heterogeneity

Our meta-analysis found consistent effects of innate immune interventions on early parasite dynamics across all three rodent *Plasmodium* species investigated (Figure 4.2). The overall effect sizes (i.e., the absolute meta-analytic mean Cohen's h) were 0.1498, 0.2321, 0.2315, and 0.2116, respectively for *P. chabaudi*, *P. berghei*, *P. yoelii* non-lethal and *P. yoelii* lethal. These effects sizes are small because a Cohen's h around 0.2 is considered a small effect and 0.5 should be reached to conclude a medium effect (Cohen, 1977). Back-transforming these effect sizes translates to a parasitaemia difference between treatments and controls at peak of roughly 6 to 12% (6.20%, 11.57%, 9.45% and 8.67%, respectively in *P. chabaudi*, *P. berghei*, *P. yoelii* non-lethal and *P. yoelii* lethal, Figure 4.2). Like Cohen's h, these back transformations refer to the magnitude, not direction, of the effects.

We detected a very low amount of heterogeneity among effect sizes for *P. chabaudi*, *P. yoelii* non-lethal and *P. yoelii* lethal ($I^2_{[total]}$ = 1.27%, 0.92% and 1.82%, respectively) which is consistent with random variation affecting measurements between studies using different approaches. This indicates that small sample sizes are more likely to account for variation in response to immune perturbations in these species rather than host, immunological, or experimental design factors. In contrast, the *P. berghei* data set contained comparatively higher heterogeneity ($I^2_{[total]}$ = 10.17%), suggesting host, immunological, and experimental design factors may underpin this variation. We examined these potential sources of variation using a series of univariate meta-regressions, which we report in the following sections (Table 4.1).

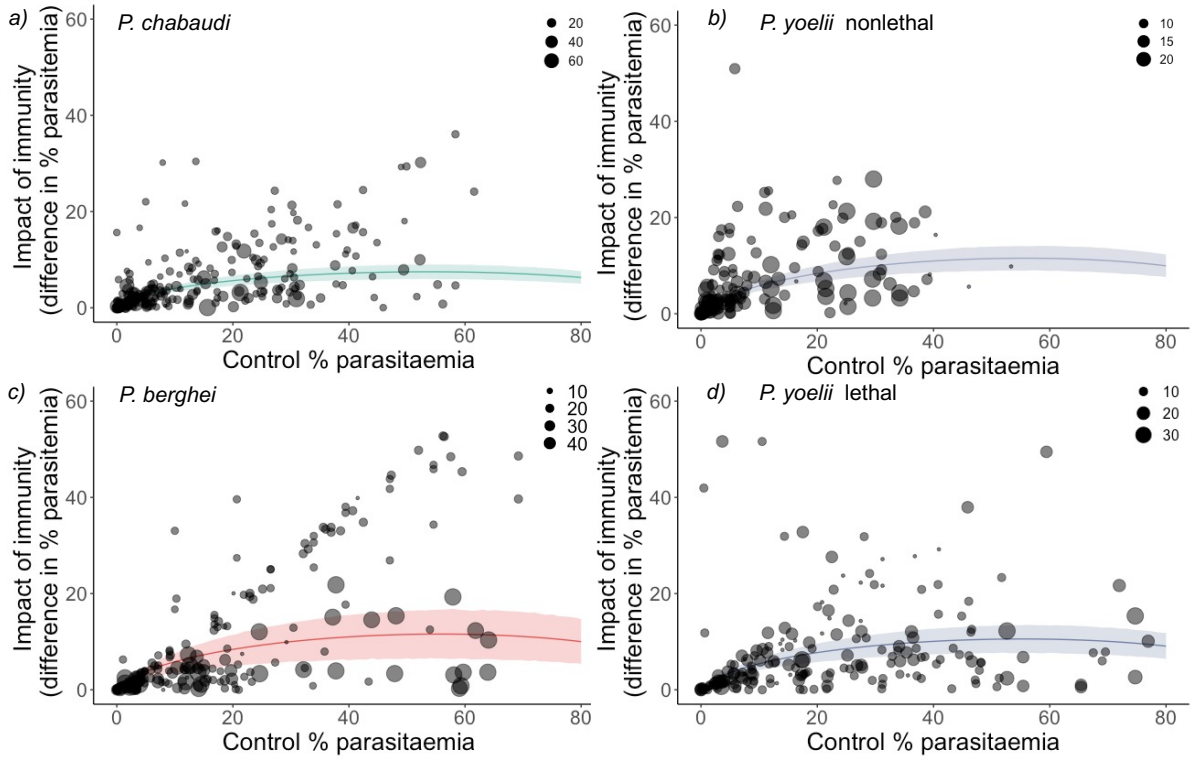


Figure 4.2. The predicted mean impact of innate immune interventions from the meta-analytic model. The impact is expressed as the absolute difference in parasitaemia in a treatment relative to its control group. Note, because some studies enhance, whereas others reduce, immune efficacy, these plots do not contain directionality, and so, do not indicate that control groups have a higher parasitaemia than treatment groups (or vice versa). Instead they illustrate the magnitude of the difference between control and treatment groups. Each panel refers to different model systems: a) *P. chabaudi* = green, b) *P. yoelii* non-lethal = dark blue, c) *P. berghei* = red d) *P. yoelii* lethal = lighter blue. The regression lines and

95% confidence bands are shown, with the size of data points reflecting the sample size of contributing studies.

Table 4.1. Comparisons of univariate meta-regression models against the null model, testing the effect of moderators in *P. berghei* infections. Delta AICc, LRT and N refer to differences in Akaike information criteria with correction, likelihood ratio test and the number of studies, respectively.

Category	Moderator	Delta AICc	LRT	p-value	N
a) Host traits	Sex	7.5026	11.6997	0.0029	32
	Age	5.5859	7.6944	0.0055	20
b) Aspects of innate immunity	Signalling position	13.3607	15.4642	<.0001	24
	Cell Lineage	2.4257	1.7713	0.4124	31
	Cytokine-chemokine, or their receptor	3.8071	0.0000	1.0000	11
	Systemic phenotype	3.4694	0.7343	0.6927	29
c) Methodology	Sampling timing	1.4336	3.5208	0.0606	31
	Parasite injection dose	0.4778	1.6094	0.2046	31
	Parasite injection route	2.0703	0.0252	0.8738	28
	Manipulation type	5.6903	9.9000	0.0071	29

Effect of host traits

We found that the impact of immune interventions declines with host age (estimate = -0.1000, SE = 0.0343, t-value = -2.9163, p-value = 0.0092, Figure 4.3; Table 4.1). For young mice (5.5 weeks), the estimated absolute Cohen's h was 0.473, which tends toward a medium effect. This constitutes up to a maximum parasitaemia difference of ~18% between control and treatment groups. Female mice exhibited stronger impacts of immune interventions than studies involving both or unknown (unreported) sexes (Figure 4.3; Table 4.1b). The average absolute Cohen's h among female mice was

0.396 which is considered a small to medium effect (Cohen 1977), and translates to a maximum difference of ~16% in parasitaemia between control and treatment. However, the lack of experiments using only male mice (N=1) precludes direct comparison between sexes.

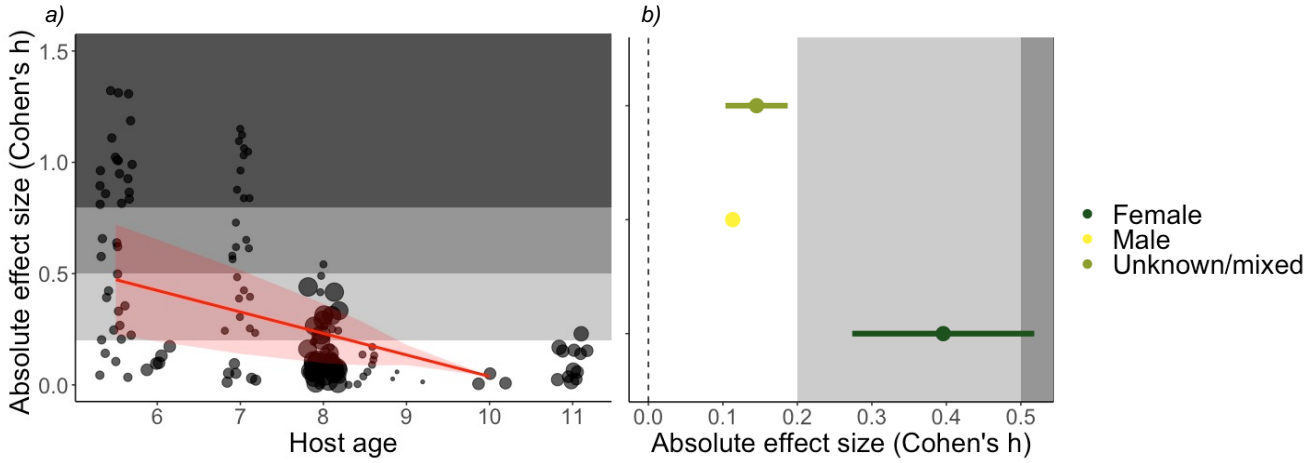


Figure 4.3. Predicted impact of host a) age and b) sex on the absolute Cohen's h effect size. a) The regression line is shown in red with a 95% confidence band, and the points represent data with their size reflecting the sample size. b) Mean absolute effect ± SEs in three sex categories (females N = 7, males N = 1, unknown N = 23). The grey shading denotes the reference for Cohen's effect size bands; small (0.2), medium (0.5), and large (0.8) (Cohen, 1977).

Effect of perturbing different aspects of innate immunity

Out of the four moderators related to aspects of innate immunity only the components position in a signalling pathway was significant, with interventions interfering with output effector mechanisms having significantly larger impacts than those modifying input signals ($F(1, 22) = 16.8373$, p-value = 0.0005, Figure 4). The average absolute Cohen's h for output was 0.466 which is considered a medium effect (Cohen 1977) and translates to a ~18% difference in parasitaemia between control and treatment groups. Other immune moderators (cell lineage, cytokines-chemokines, and systemic phenotype) did not significantly explain heterogeneity in the meta-analytic mean (Table 4.1).

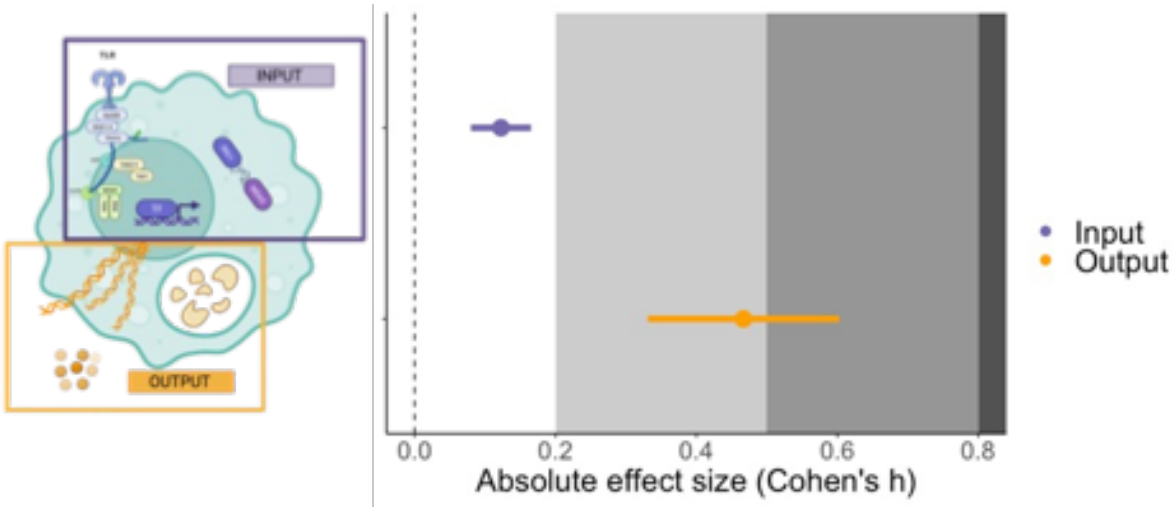


Figure 4.4. The cartoon (left) illustrates our division of roles in signalling cascades: input (purple), refers to factors with an upstream signalling position (e.g. pattern recognition receptors PRR and transcription factors), and output (yellow), refers to downstream factors (e.g. effectors like NO, proteases and nets) (left). The figure (right) shows the mean and ±SE effect size input and output interventions in *P. berghei*. The grey shading denotes the reference for Cohen's effect size bands; small (0.2), medium (0.5), and large (0.8) (Cohen, 1977). The left cartoon was created using BioRender.com.

Effect of perturbation methodologies

Of the four factors describing aspects of experimental designs, only the type of manipulation explained significant heterogeneity. Drug induced perturbations to innate immunity generated the largest impact (Figure 4.5). However, the average Cohen's h was only 0.386, which is considered a small to medium effect (Cohen 1977), and translates to a ~15% difference in parasitaemia between control and treatment. Furthermore, the impact of drug induced manipulations to immunity was significantly higher than mixed approaches ($\chi 2 = 4.561$, p-value = 0.0327) and genetic modification (GM) of hosts ($\chi 2 = 10.4520$, p-value = 0.0012). Finally, we found no moderating effects of sampling regime, parasite inoculation dose, or injection route (Table 4.1).

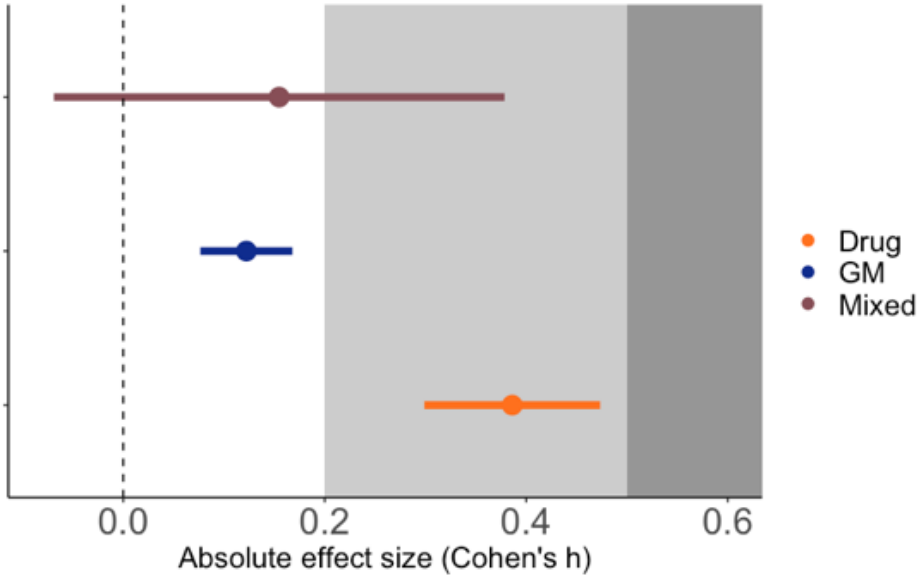


Figure 4.5. Mean ±SE effect size in levels of moderator "manipulation type" for *P. berghei*. Mixed= more than one manipulation type N=3 studies, GM=genetic modification N=13, Drug=agonist or antagonist N=13. The grey shading denotes the reference for Cohen's effect size bands; small (0.2), medium (0.5), and large (0.8) (Cohen, 1977).

Assessment of publication bias

In metanalytic approaches publication bias allows us to know if the published literature from where our study samples come is skewed; for example publishing only positive and big effects. As illustrated by Figure 4S2 and the regression and rank tests (Table 4S3), we detected little evidence of publication bias in *P. chabaudi*, *P. yoelii* lethal and *P. yoelii* non-lethal. However, we found a potential indication for publication bias in the *P. berghei* dataset (Table 4S3), so we carried out a sensitivity analysis by removing the three articles that contributed most to asymmetry. Removing these studies made little difference to the overall mean effect size (Figure 4S3) indicating the analyses presented above are not affected by publication bias.

Discussion

We present a broad ranging meta-analysis of studies spanning several decades of using experimental perturbations to identify the roles of innate immunity in the early phase of malaria infection. Because some studies perturb immune function in ways that facilitate parasite replication, whereas other decrease parasitaemia, we quantify

effect sizes on the absolute scale, which reports the magnitude (not the direction) of the difference between control and treatment groups. Overall, we reveal effect sizes (Cohen's h) that are considered small for the impact of perturbating components of the innate immune system on parasite replication for all four data sets spanning three species (Figure 4.2)(Cohen, 1977). The effect sizes range from 0.149 and 0.233 which translates to a parasitaemia difference of roughly 6-12% between the control and immune-manipulated groups at the peak of infection, with *P. berghei* infections at the upper end of this range. We also reveal that for *P. berghei,* the impacts of immune interventions are larger when younger, or female, mice are used as hosts (Figure 4.3), and greatest when effector molecules or networks involved in innate immunity are perturbed (translating to an 18% difference in peak parasitaemia between control and treatment groups) (Figure 4.4). In contrast, there is extremely low heterogeneity for *P. chabaudi and P. yoelii,* which means variation in the impacts on immune manipulations on infection dynamics is better explained by stochasticity due to small sample sizes than by different experimental details.

The small effect sizes across all data sets has several non-mutually exclusive explanations. It may reflect redundancy in a complex system. Innate immune responses against malaria are underpinned by a complex network of signalling pathways with considerable functional resilience (Gazzinelli et al. 2014; Gowda and Wu 2018). Thus, the innate immune system may compensate and partially restore the function following perturbation (Nish & Medzhitov, 2011). The observation that effect sizes are larger when effector molecules and networks (outputs) are perturbed is consistent with the expectation of greater functional redundancy in upstream elements of pathways, including receptors (El-Brolosy & Stainier, 2017). Similarly, the smaller effect sizes observed in older or GM hosts may be due to age conferring robustness to perturbationand genetic knockouts are often compensated in immune systems (El-Brolosy & Stainier, 2017). That the innate immune system is intertwined with physiology and development adds to the difficulties in dissecting out the role of innate immune components in controlling parasites. Thus, the effect sizes we measure may simply reflect the lower bound of the overall effect of a component of the innate immune system. However, redundancy and robustness cannot explain the non-significant impacts of manipulating major components of the innate system (e.g. depletion of the myeloid lineage). That such global suppression of innate function has

minor, if any, impacts *in vivo* agrees with some *in vitro* studies where no effects of similar conditions are detected (Stevenson & Riley, 2004). Thus, an alternative explanation is that innate immunity has minor direct impacts on parasite replication and instead, operates to mobilise elements of the adaptive immune response. For example, malaria infection triggers interferon-stimulated inflammation and emergency myelopoiesis (Götz et al., 2017; Langhorne, 1994). This response is also elicited by viral infection, injury, or trauma which suggests it is not specifically directed to early parasite control. By leading to the recruitment of monocytes and memory T cells to the spleen, well controlled inflammatory response may serve to bring innate and adaptive immune cells together to co-ordinate the killing and clearance of parasites (Langhorne, 1989; Riley et al., 2006). Meta analysis could establish the importance of mobilisation using complex multivariate analysis that test for an interaction between time and trafficking but the small numbers of studies in the literature, which also have small sample sizes, preclude this (Table 4S2).

We detected low heterogeneity among studies, indicating that the majority of variability across publish studies are attributable to differences in the sample size used by different studies (Higgins, et al., 2003). Low heterogeneity was unexpected because our datasets include mice of diverse genetic backgrounds (e.g., C57BL/6 Stevenson 1989, 129Sv Kim et al., 2012 and A/J Stevenson 1989) that show diverse infection phenotypes (Craig et al., 2012; de Niz & Heussler, 2018). However, low heterogeneity is commonly found among *in-vivo* studies, in which sample sizes are typically small (and hence studies suffer low precision) due to the ethical considerations of using animal models (Vesterinen et al., 2014). As such, larger scale experiments, are needed to identify sources of mixed results in the literature, particularly in *P. chabaudi* and *P. yoelii* infections, where we detected <1% heterogeneity. Why heterogeneity among studies was detected for *P. berghei* (~10% variation was not attributable to sample size variability) is unclear. *P. berghei* is not used more often than other species and *P. berghei* experiments do not have larger sample sizes, suggesting a biological explanation. It is possible that because *P. berghei* infections are short (i.e. terminated before cerebral malaria develops), there are less opportunities for confounding factors, such as RBC depletion, to confound the impact of innate immunity on parasite replication compared to other species. Additionally, compared to other species, hosts might be more likely to resist than tolerate *P. berghei* (Olatunde et al., 2022). While

this could reveal a greater impact of innate immunity, it may not reflect malaria species more broadly or provide a good model for human malaria infections.

We did not detect a moderating effect of either the timing of sampling or initial infection dose on the impact of immune interventions. A lack of impact of timing could, theoretically, arise because the possible difference between control and treatment groups is smaller at the lower boundary. For example, when parasitaemia is low early on in infections, there is less scope for parasitaemia to be reduced by immune augmentation than at the higher parasitaemias reached later in infections. Fortunately, standard effect sizes, like Cohen's h, allow for examination of effects free from this constraint making our inference about the timing of sampling robust (Cohen, 1977). The lack of a dose effect was unexpected because most mathematical models capturing the effects of innate immune mechanisms in humans invoke links between parasite densities (dose) and immune responses (Camponovo et al., 2021). Perhaps by assuming strong impacts of innate immunity on parasites, the development of tolerance mechanisms (which are less dependent on parasite burden) has been overlooked (Nahrendorf et al., 2021).

We also identified host sex and age as moderators of innate immunity in *P. berghei* infections, with female and younger hosts generating stronger impacts on immune interventions (Figure 4.3). The sex-dependent immune response against malaria is consistent with classic findings that males are comparatively immunocompromised due to the higher production of testosterone that downregulates the immune response (Klein, 2004). The role of immunosenescence – i.e., reduced immune efficacy with age against malaria infections remains an open question. A recent systematic review concluded that immune responses generally elevate with age (Felizardo et al., 2018) while some report that older hosts are more susceptible to *P. yoelii* infections (Sorci et al., 2021). It is important to note that our study only includes young to middle-aged, sexually mature mice (from 5.5 to 11 weeks). Thus, while our result indicates that younger hosts react more strongly to innate immune interventions, more experimental data are needed to conclude the impact of immunity in juvenile and older mice. As has been remarked elsewhere (vom Steeg et al., 2019), we noticed that the reporting of host traits tends to be overlooked. For example, many studies in our dataset did not

explicitly report the sex or used only one sex (n = 79 and 61, respectively out of 140 studies).

In addition to better reporting of host characteristics, larger sample sizes, and a broader understanding of how immune manipulations perturb host physiology more broadly, we also advocate for improvements in data reporting. Parasitaemia (the proportion or % of infected RBCs) is widely used as a proxy of parasite burden. However, this metric is problematic for several reasons. Most importantly, the proportion of infected RBC is confounded by a change in its denominator, i.e., the number of RBCs. As malaria infection progresses, parasites exploit and deplete RBCs, and host-mediated mechanisms clear RBC in substantial numbers (e.g., phagocytosis of both infected and uninfected cells) and suppress RBC production. Thus, longitudinal parasitaemia data provide incomplete information on rates of parasite replication and death – i.e., it is impossible to distinguish whether top-down (e.g., immune responses) or bottom-up (e.g., indiscriminate RBC clearance and RBC replenishment) mechanisms underly the observed patterns. Addressing this can be achieved simply including hemocytometry or flowcytometry to count RBC and by reporting counts of both RBC and parasites at each sampling point. While this is not routine, there is precedent to provide a fuller picture of infection dynamics; among the *P. chabaudi* experiments in our dataset, all but 4 articles report paired parasite RBC counts.

Conclusions

Our meta-analysis summarised four decades of research using rodent models to probe malaria innate immune responses. We detected a small overall effect of innate immune interventions on malaria infection dynamics, reveal that small sample sizes can explain much of the variation in outcomes of different studies for *P. chabaudi* and *P. yoelii*, and suggest that manipulating of innate immune effectors by drugs in young female hosts have the largest impacts on *P. berghei* infection dynamics. Why the impacts of different approaches varies between species is unclear and raises questions about how to translate findings from rodent models to human parasites. The immune response across mammalian taxa is highly conserved (Shay et al., 2013), but differences between human and rodent *Plasmodium* species exist, including that

rodent *Plasmodium* complete asexual replication in half the time it takes *P. falciparum*. Perhaps the capacity of rodent *Plasmodium* to replicate to much higher densities causes resource limitation to play a larger role in limiting parasite burden. No study system marries ecological realism, translational value, and tractability. Yet, a better integration of experimental immunology with mathematical modelling can help to challenge the assumptions of models, provide alternative hypotheses for experimental testing, and uncover generalities across host and parasite taxa. Combining a more integrative approach with larger scale experiments that report more comprehensive data should help resolve conflicting reports on the role of innate immunity in malaria infections.

Chapter 5. General discussion

This thesis aimed to use an eco-evolutionary framework to explore host-parasite interactions, focussing on three poorly understood traits of malaria parasites. To maximise ecological relevance and tractability, I studied *Plasmodium chabaudi,* a rodent malaria model. Here, I summarise my key findings and their broader context, before discussing the limitations of my approaches and providing directions for future research.

5.1 Key findings and broader context

In Chapter 2, I discovered that parasites transition from the first phase of infection in the liver of the mammalian host, to the next phase in the blood, in a manner unrelated to host rhythms. Specifically, parasites gradually accumulate in the blood over a prolonged window (spanning at least ~16 to 20 hours) and patterns of accumulation in the blood differ between parasite genotypes. This result was counter to expectation – suggesting parasites are missing the opportunity to begin the IDC at the best time of day (phase) with respect to host rhythms. However, this can be explained by additional results revealing that the manner that parasites initiate the blood phase is selectively neutral. I conclude that the parasites' ability to rapidly reschedule the IDC when its timing is perturbed has evolved to ensure the IDC is rapidly aligned with host rhythms following egress from the liver. In principle, this could be tested by assessing rhythmicity of the first few IDCs following egress, but this is not currently feasible for parasites at very low densities which need to be staged by microscopy. Furthermore, that host rhythms do not directly affect the ability of parasites to establish infections, suggests that the time of day malaria infections at initiated by blood-feeding mosquitoes does not matter to parasites (but we did not assess the longer term consequences for hosts in terms of disease severity). If so, this suggests that reports of mosquitoes changing the time of day they bite (Sangbakembi-Ngounou et al., 2022) (to evade insecticide treated bed nets), will not enhance parasite transmission beyond simply making vectors available or via altering parasite-vector interactions. However, whether host rhythms influence other activities of parasites in the liver phase, or the

ability of sporozoites to reach the liver, remains to be tested. Thus, my results may highlight that assessing the impacts of host rhythms should be undertaken across the whole life cycle (i.e. from the skin to the liver and blood, and throughout the different tissues colonised in vectors).

While feeding related rhythms are the most apparent in the activities of liver cells, innate immune responses also operate in the liver and are likely to be rhythmic. Such responses may impact parasites during the liver phase or prime responses mounted in the blood that parasites encounter once egressed. If so, parasites may not be able to avoid these rhythmic dangers by egressing at a certain time of day if this would cause the IDC schedule to be out of synch with feeding-fasting rhythms. Thus, arrhythmic egress may dilute exposure to these dangers whilst also minimising the degree of IDC scheduling that needs to occur. A better understanding of immune rhythms during infection is needed to make specific testable predictions for this hypothesis (Hunter et al., 2022), by this I mean predicting what time of day would be beneficial/detrimental for the parasites with respect to immune rhythms in the liver or caused by the liver but active in the blood stage. For example, the magnitude of inflammatory responses mounted by mice against *Leishmania* parasites depends on the time of day, creating bigger lesions when infection is initiated during the end of the dark phase (i.e. late at night (Carvalho Cabral et al., 2019)). Whereas the opposite occurs for *Salmonella* infections; inflammation is greater when mice are challenged during the early resting phase (Bellet et al., 2013). However, except for *Trypanosoma brucei* and Leishmania (Rijo-Ferreira and Takahashi, 2022), most studies have been conducted using parasites with a less complex lifecycle than *Plasmodium* and/or lack control over their rhythms. Therefore, the observed rhythms and their consequences reflecting host-imposed characteristics on parasites that are passively affected by host rhythms. In addition, some parasites can manipulate host rhythms (Carvalho Cabral et al., 2019) thus potentially interfering with the impact of host rhythms (Westwood et al., 2019). Malaria infection alters locomotor and temperature rhythms of mice, but it is unknown whether parasites are manipulating host molecular clock components (TTFL) or this is a by-product of other malaria symptoms. However, it is possible that during their liver stage parasites can impact host cell clocks or their outputs because liver stage parasites can manipulate other aspects of liver cell physiology, including

autophagy, metabolic and trafficking pathways (Sturm and Heussler, 2007; Vijayan et al., 2022; Zuzarte-Luis and Mota, 2020).

In chapter 3, I ventured into the role of host rhythms in parasite sequestration. I found that the role of host rhythms in parasite sequestration is subtle and variable across tissues. Overall, host rhythms affect parasite abundance in the liver and lungs but not the spleen. In general, infecting a host in which the phase relationship between SCN-driven and feeding-fasting rhythms is disrupted facilitates sequestration, and most parasites sequester when misaligned to one of these host rhythms. The patterns of abundance within some tissues suggest that the time of day sequestration normally occurs (in the dark phase, at night for murine hosts) is the most challenging for parasites. If so, sequestration is associated with intrinsic costs that must be substantially offset by benefits (e.g. of evading splenic clearance). Previous studies have found that removing the spleen causes parasites to stop sequestering and selection *in vitro* reduces the production of the exported antigenic variants responsible for adhesion (Bachmann et al., 2009; Gilks et al., 1990). That sequestration is rapidly lost in environments where parasites do not face dangers from the spleen or the immune system suggests it is intrinsically costly.

My findings also suggest that hosts with disrupted rhythms are less able to control sequestered parasites. It is also possible that hosts with disrupted rhythms make sequestration easier, for example, by providing more sequestration receptors. I propose this is unlikely because whether the abundance of sequestering parasites within a tissue is mediated by the availability of receptors (i.e. can they ever be limiting?) is unknown, and my data do not suggest there were fewer parasites in the circulation in hosts with disrupted rhythms. Instead, perhaps conflicting time cues from the SCN and peripheral clocks affect the rhythms of immune factors, reducing their efficacy against sequestered parasites. Yet, the variation I observed in sequestration patterns is not sufficient to impact on the replication success of the focal IDC cohort. This is unlikely to be because sequestration is a selectively neutral trait. Instead, I propose that the benefits of sequestration increase as infections progress, when splenic and other innate immune defences are fully activated. And that sequestration appears to be a trait parasites are constrained to express every IDC, so they sequester needlessly (or perhaps in a slightly costly way) within the first few IDC. This hypothesis

is in keeping with the suggestion that sequestration is a bystander consequence of antigenic shift (Sherman et al., 2003). More broadly, my findings and approach demonstrate that gaining a better understanding of what governs opportunities for sequestration and the resulting costs and benefits is tractable. In keeping with the need to study the impact of host rhythms across the parasite lifecycle to understand the role of host rhythms for liver stage parasites, the findings from future studies of sequestration should also be integrated across all tissues the parasite uses.

In Chapter 4, I tested the conventional wisdom that innate immune responses play an important role in controlling parasite replication in the early acute phase of blood phase infections. My meta-analysis revealed modest impacts of perturbations of innate immune factors on parasitaemia, and that few generalizations can be made due to species-specific patterns and study-specific characteristics (such as experimental tools and host biological factors). Only finding heterogeneity across the results of different studies for *P. berghei* was unexpected, and it does not appear to be driven by *P. berghei* having greater statistical power compared to studies with the other species. Instead, I suggest further research that focuses on this parasite's immune evasion characteristics or whether it elicits stronger innate responses, might be key to explaining such phenomena. In addition to the problems of using parasitaemia discussed within Chapter 4, it is also the case that parasitaemia cannot differentiate between immune and resource-mediated control of proliferation. However, the early acute phase of infections in well fed and naïve mice might not be subjected to severe resource limitations. Nonetheless, the role of immune perturbations has mainly focused on top-down control (i.e. immune killing) of proliferation, but it is increasingly being recognised that innate responses can also interact with bottom-up effects (i.e. resource availability), for example, due to their roles in haematopoiesis and metabolism. Important bottom effects include the availability of RBC at the preferred age and parasites' ability to invade them. Unfortunately, there are far fewer studies of resource perturbations than immune manipulations, likely precluding a meta-analysis on this topic.

Other fundamental messages emerged from the work in Chapter 4. First, the majority of relevant studies do not present their results in a complete enough or appropriate

manner to use in a meta-analysis (thus, lack added value). The field would benefit from raising standards in data reporting and statistical analysis to maximise the utility of data; the popularity of meta-analysis in evolutionary ecology provides proof of principle for the value of this approach as well as guidance for data reporting (O'Dea et al., 2021). Second, many mathematical models of within-host dynamics may assume a stronger impact of early immune responses than are observed through manipulating immune mechanisms. Discrepancies between modelling and experimental approaches indicate aspects of the underlying biology are not understood and risk models being based on inaccurate biological assumptions. Finally, if the ecology of within-host dynamics is misrepresented in a model system (mice) this means that less tractable systems, like human malaria infections, are even more challenging to understand.

5.2 Limitations of my approaches

Whilst rodent malaria models are generally very tractable, because I explored traits that are rarely studied the tools available are not perfect so I had to make compromises to my approaches in all projects.

First, In Chapter 2, it is not possible to directly assay egress from the liver, so I had to assume that parasites detected in the blood represent recent egress. The molecular techniques I employed to count parasites in the blood means that our ability to detect is bounded by the sensitivity of the assay. Thus, an assay with higher sensitivity might reveal that accumulation in the blood starts at certain times of day. Though, I note this is unlikely because there were no statistically borderline trends across treatment groups that would be bolstered by more sensitive assays. Furthermore, given that the timing of the IDC is a fitness-related trait, the pattern of accumulation in the blood, not necessarily egress itself, is a key phenotype exposed to natural selection. Another limitation is not being able to measure the number of infected hepatocytes (because it requires destructive sampling), nor the size of sporozoite inoculum. Thus, host rhythms may have affected the productivity of infected hepatocytes. However, previous studies reveal that differences in sporozoite inoculum only change the magnitude of blood dynamics, not its timing (De Niz et al., 2016).

Second, the biggest obstacle in Chapter 3 is the use of parasite abundance in tissues as a proxy for sequestration. This was necessary because the ideal approach (perfusing organs) is not high throughput enough for the sample sizes of eco-evolutionary experiments. I also note that it is common for studies to use proxies for sequestration, including proximity to vascular walls or locating parasites in bulk using bioluminescent marked parasites, or by comparing parasite densities in tissues relative to peripheral blood.

Third, I was not able to assess parasite multiplication rate as the response variable in the meta-analyses because it is not commonly reported. Instead, I had to use parasitaemia, which can introduce bias; if red blood cell densities fluctuate, parasitaemia is not independent of anaemia (RBC dynamics). The parasite multiplication rate (PMR) is preferable because it includes several parasite biological traits like invasion efficiency and, the number of merozoites per schizont (burst size). These biological traits influence the proliferation rate of infection, thus making it possible to disentangle which of these traits immunity is acting on (e.g. revealing if immune responses block RBC invasion efficiency rather than burst size or clearance). Calculating the PMR could give new insights into the impacts of host immunity (Gnangnon et al., 2021).

5.3 From mice to humans

Beyond the limitations particular to the methods used in each chapter, I am also aware of the inherent limitations of rodent malaria model systems. The rodent host (laboratory mice) used in this thesis is not the natural host for *Plasmodium* malaria species which were isolated from several species of thicket rat (e.g *Grammomys poensis* and *G. surdaster*) (Killick-Kendrick and Peters, 1978). Unfortunately, Thicket rats are not amenable to rear in lab colonies, and they are not tractable models because their physiology is not well characterised and there are no genetic tools available for them. How closely infection dynamics correlate between laboratory mice and thicket rats is unclear. For example, *P. chabaudi* genotype ER dynamics in thicket rats is very similar to those in C57BL6 hosts (Conteh et al., 2020). But, a consistent finding in experimental mouse models - but not in the thicket rats - is a decrease in white blood cells (WBC) during the acute phase paired with a decrease in haematocrit

(Conteh, 2020). This difference may be important in translating findings to natural infections for which host and parasite have coevolved because the same sporozoite challenge is insufficient to generate protection in thicket rats but is sufficient for laboratory mice (Conteh et al., 2017). Such a difference in outcome might explain mixed success in the use of attenuated sporozoites for vaccinating humans (Bijker et al., 2015). Nonetheless, similar immune phenotypes are reported for *P. chabaudi* and *P. falciparum* (Stephens, et al., 2012). Furthermore, the tools available for perturbing aspects of lab mouse biology (such as impairing the TTFL using Per1/2 knockouts) can be deployed in an ecologically relevant setting (i.e. *in vivo),* unlike for *in vitro* models and other vertebrate hosts.

While the aim of my thesis was to investigate basic parasite ecology and test for proof of principle of concepts, the basic principles stemming from life history theory do translate to human malaria parasites. For example, the well-understood sex ratio and conversion rate strategies that *P. chabaudi* deploys are consistent with observations of *P. falciparum's* strategies (Schneider and Reece, 2021), and have been validated with data from natural infections (Early et al., 2022). This is not surprising because the same strategies have evolved across diverse taxa (including mammals, birds, insects, plants, and viruses), so *P. falciparum* is unlike to be an exception to such general rules. Thus, our findings: that host time of day has no impact on the start of the blood phase, the role of disrupting host rhythms in parasite abundance across organs, and the impact of early innate responses, should broadly apply to rhythmic *P. falciparum* infections in humans. Thus, the knowledge generated in this thesis can lead to practical applications. For example, uncovering parasite strategies and the details of their limits and constraints may reveal novel targets for interventions and how to make existing interventions more robust against unfavourable parasite evolution.

5.4 Future directions

This thesis opens exciting new questions to be addressed and illustrates how an interdisciplinary approach can be used. Key outstanding questions stemming from each chapter are summarised in Table 5.1. From this table, I will expand on one question from each data chapter. 1) Question 4, Do parasites begin adjusting the IDC

schedule as soon they egress from the liver and invade RBC?, Table 5.1. This question will provide a key mechanistic understanding of how parasites establish rhythmic asexual replication during the blood stage, this will lead to knowing how they achieve coordination and how soon. Derived from chapter 2, we know parasites do not schedule their transition from the liver to match specific host rhythms, but these rhythms are known to matter during the blood stage (O'Donnell et al., 2011; 2013). So exploring how soon after their transition will provide further clues about this parasite strategy and might provide a window for timing interventions. 2) Question 11, Do parasites ever experience limitations in the availability of sequestration sites? Table 5.1. Little is known about the ecology of parasites within tissues, we could imagine parasites competing for sequestering sites if the benefits between sites differ. Moreover, if they do not get saturated then the assumption that oscillations make availability sites change would be irrelevant. In chapter 3, I was limited to the number of parasites therefore if parasite densities matter for benefits gained from sequestration this could not have been assessed using my experimental design. I think information obtained from answering this question will be key for disrupting parasites either saturating sites and/or forcing parasites to pay higher costs by remaining in circulation or adhering to less favourable environments and could be exploited in multiple coinfections. Finally, 3) Question 18, Are immune rhythms important in the early acute phase; would innate immune perturbations have a stronger/weaker effect in clock disrupted mice, and depend on whether the parasite has a synchronous or an arrhythmic IDC? from Chapter 4 I would like to link the chronobiology discipline into the immune effectors and our meta-analysis results, including host rhythmic immune response and the interaction with rhythmic parasites, previous literature had similar musings into whether synchronous/asynchronous species link to the immune response phenotype (Taylor-Robinson & Phillips, 1998). If the host immune response is context dependent and we focus mainly on the early phase this translates to answering do arrhythmic (i.e. continuous) bursting lowers immune activation or the other way around? By creating arrhythmic situations in rhythmic strains and the opposite could test for this effect in vivo but is limited by the parasite's natural scheduling time. Thus, measuring whether this arrhythmic/rhythmic strategy makes host response more effective (e.g. higher immune activation/ mobilisation of immune cells) differ between these two parasite strategies, for example limiting inflammation

by spreading the bursting in prolonged windows whereas the synchronized bursting parasites probably evolved ways to limit inflammation by other means.

Table 5.1. Future directions, colour coded as follows: Chapter 2 (purple), Chapter 3 (orange), and Chapter 4 (green).

Chapter 2: Rhythms and the liver phase	
1	Does host time of day impact on how well sporozoites can migrate to hepatocytes and invade them?
2	Do liver rhythms, relating to redox and/or nutrients impose constraints, or offer opportunities to liver phase parasites?
3	Do parasites follow developmental rhythms when replicating within hepatocytes?
4	Do parasites begin adjusting the IDC schedule as soon they egress from the liver and invade RBC?
5	Why do parasites not deploy the time-keeping mechanism they express in the IDC? Are there costs associated with assessing and responding to time cues unnecessarily? Does this suggest parasites do not have a constitutively expressed oscillator?
6	How much plasticity is there in the activities of liver phase parasites?
7	Do other genotypes follow the same blood accumulation patterns as AJ or AS, and how much genetic variation is there for these patterns?
Chapter 3: Rhythms and sequestration	
8	What are the intrinsic costs of sequestration?
9	What are the benefits of sequestration, in addition to evading splenic clearance? And do non-sequestering species miss out on these benefits?
10	Do the costs and benefits of sequestration vary across tissues, and during infections?
11	Do parasites ever experience limitations in the availability of sequestration sites?
12	What are the consequences for hosts of parasites sequestering at different times of day across tissues?
13	Do asexual and gametocyte committed parasites have different requirements from the sequestration microenvironment?

Chapter 4: Immune impacts on early infections	
14	Are species differences in the impact of innate immunity explained by differences in parasite ecology (e.g. RBC age preference), and/or the immune responses they stimulate?
15	What does limit/control parasite multiplication rate in the early acute phase?
16	How should studies quantify and disentangle the redundancy and pleiotropic effects of the immune system?
17	Are the innate immune responses activated in the early acute phase intended to establish tolerance? What determines the host disease tolerance/resistance threshold among parasite species?
18	Are immune rhythms important in the early acute phase; would innate immune perturbations have a stronger/weaker effect in clock disrupted mice, and depend on whether the parasite has a synchronous or an arrhythmic IDC?
19	Do the modest impacts of innate immune factors on early infection dynamics hold for other kinds of severe infection?
20	How can parasitology and immunology be encouraged to measure and report infection dynamics in more detail?

5.5 Conclusion

My thesis showed that *Plasmodium* parasites' evolutionary strategies can be stage-dependent (liver egress is arrhythmic but the IDC is rhythmic), and that a hallmark of *Plasmodium* immune evasion (sequestration) might involve multiple non-exclusive selective drivers beyond just escaping host immune response. Finally, there are few moderate and clear impacts when perturbing the innate immune system on early acute stage infection dynamics, though poor quantification of studies limits inference. As an evolutionary ecologist, it is an exciting moment to apply novel frameworks to explore why parasites exhibit certain traits and uncover their strategies. In the future, such understanding should improve interventions against parasites, by for example, tackling traits that are highly constrained to minimise the speed of resistance evolution.

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Supplementary Information

Table 2S1. Citations of R packages used for statistical analysis.

R packages implemented in Chapter 2
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Table 4S1. Explicit exclusion criteria. Table displays the eligibility criteria employed based on PICO defined elements (McKenzie et al., 2022) .

	Explicit exclusion criteria
Subject	The subject is not related. Indirectly testing the effect: correlative or speculative mechanism to immune function. Experiments have no control and treatment groups (for example host strains labelled as susceptible vs resistant)
Type	Not a research article (review, opinion, protocol, conference report). Article used data from another in the primary literature Not in English
Comparator	Uses parasite species that is not one of *P. berghei, yoelii, chabaudi, vinckei* uses a non-mouse host species. Infections initiated from a phase other than the blood stage (liver or mosquito derived infections) Co-infected or multiply infected hosts Uses genetically modified parasites (GMO) with a replication rate phenotype, or a mix of parasite antigens that activate the immune response, or attenuated parasites. Uses hosts previously challenged with *Plasmodium* parasites or their antigens
Intervention	Involves *in vitro* or *ex vivo* approaches Not directly testing a component(s) of the innate immune response (e.g. by using tools removing adaptive immune function) that manipulate adaptive immune components)
Response	No quantification of parasite dynamics during infections (e.g. only point estimate of difference in parasite performance between treatment and control) Day post infection information not included for parasite performance estimates Data collected after the perturbation is expected to have exerted its influence on parasite performance over 7 to 10 days p.i (first week of infection) When article shows effect in selected points through infection dynamics to compare without justifying the selected points Data not suitable to relate to parasitaemia

Table 4S2. The table shows the number of studies per moderator variable. Labels for levels within moderators: signalling (in = input, out = output), Systemic function (inflam = inflammatory, reg = regulatory, traff = trafficking), Cytokines chemokines / receptors (cc= Cytokines/ Chemokines, rec= receptors) Injection route (Ip = intraperitoneal, Iv = intravenous), Manipulation (GM= genetic modification, drug = drug, mixed = any combination). Pc = *P. chabaudi* , Pb = *P. berghei*, PyL= *P. yoelii* lethal, PyNL= *P. yoelii* nonlethal.

Immune factors			Methodology	Host factors
Within cell	Systemic function	Cell		

Parasite	Signalling position	Cytokines/ chemokine or receptors	Inflammatory Regulatory Trafficking	Lineage	Manipulation type	Injection route	Sex	Age
	46	37	47	57	61	43	61	52
Pc	18 in 28 out	26 cc 11rec	21 inflam 26 reg	9 lymphoid 15 myeloid 33 both	5 mixed 27 drug 29 GM	37 Ip 6 Iv	4 male 21 female	NA
	24	11	29	31	29	28	32	20
Pb	19 in 5 out	10cc 1 rec	16 inflam 12 reg 1 traff	1 lymphoid 7 myeloid 23 both	3 mixed 13 drug 13 GM	25 Ip 3 Iv	1 male 7 female	NA
	19	10	17	23	23	22	23	22
PyL	12 in 7 out	7 cc 3 rec	10 inflam 3 reg 4 traff	1 lymphoid 2 myeloid 20 both	1 mixed 8 drug 14 GM	7 Ip 15 Iv	1 male 18 female	NA
	16	8	17	23	24	22	24	20
PyNL	11 in 5 out	4 cc 4 rec	10 inflam 7 reg	1 lymphoid 6 myeloid 16 both	1 mixed 13 drug 10 GM	12 Ip 10 Iv	1 male 8 female	NA

Table 4S3. Table shows funnel plot asymmetry. Two common meta-analytic tests were conducted to assess publication bias (Rank tests and Egger's test).

	Rank correlation test for funnel plot asymmetry	Egger's test (regression to inverse variance)
P. chabaudi	Kendall's tau=-0.0022, p-value=0.9612	Intercept=-0.0212, SE=0.0518, t=-0.4100, df247, p-value=0.6821
P. berghei	Kendall's tau=0.2051, p-value < .0001	Intercept=0.2747, SE=0.1011, t=2.7180, df188, p-value=0.0072
P. yoelii Lethal	Kendall's tau=0.2051, p-value < .0001	Intercept=0.0454, SE=0.1276, t=0.3557, df190, p-value=0.7225
P. yoelii Non lethal	Kendall's tau=-0.0438, p-value=0.4827	Intercept=-0.0194, SE=0.1466, t=-0.1320, df137, p-value=0.8952

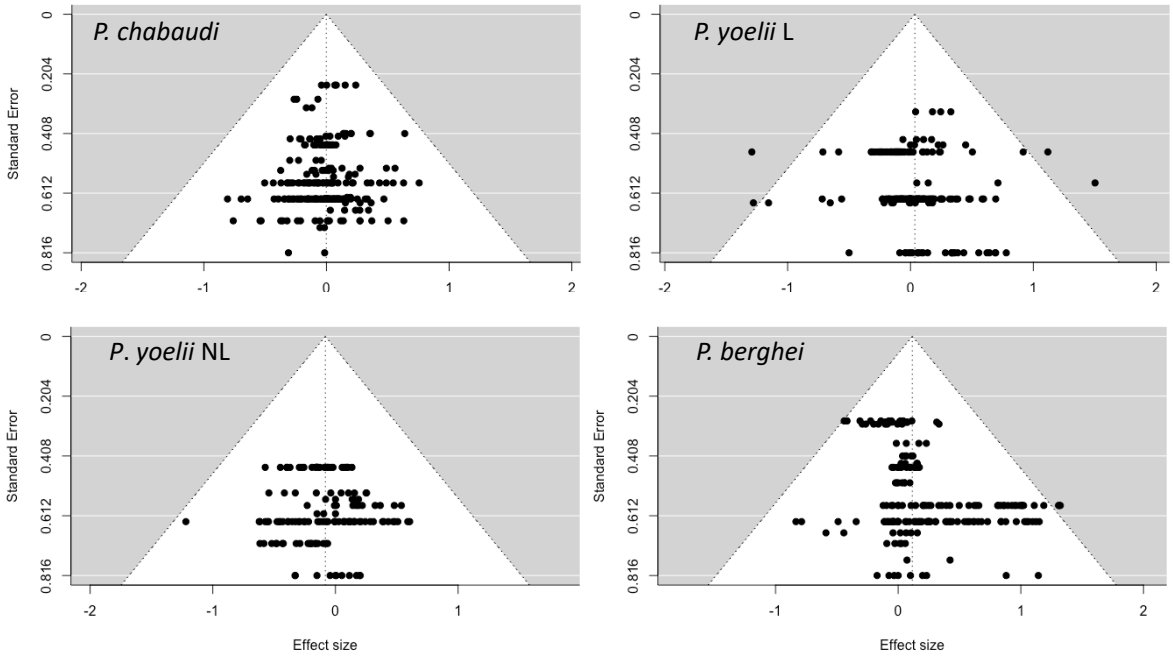

Figure 4S1. Inverted funnel plots. SE by raw effect size (Cohen's h). Triangle delimits 95% Confidence Intervals. Labels, L= Lethal, NL= nonlethal. The plots display funnel symmetrical distribution of the data, showing higher precision with smaller error, thus the funnel shape. Unlike *P. berghei* plot, a few studies are showing a positive effect unlikely to be by chance.

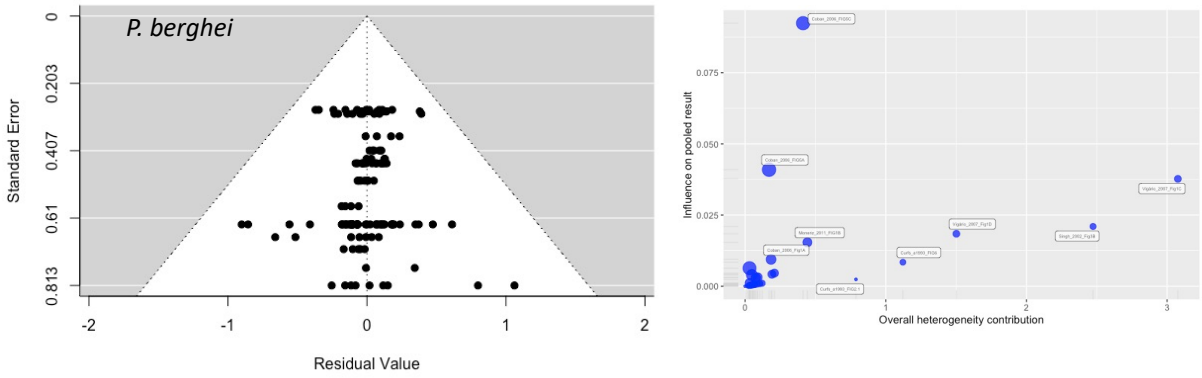

Figure 4S2. Modified funnel plot from *P. berghei* after removing three influential points. "Boujat" graph showing mean effect size per study (right), the 3 points removed are those with higher influence overall effect and heterogeneity contribution. After removing these points the tests indicate no longer bias, regression to inverse variance Egger's test, intercept = 0.1038, se=0.0854, t=1.2151, df=127, p=0.2266, 95%CI -0.0652- 0.2728.

Additional file 1

Primary data included in meta-analysis of Chapter 4

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