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A song for the unsung: The relevance of *Plasmodium vinckei* as a laboratory rodent malaria system

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ABSTRACT

Rodent malaria parasites (RMPs) allow the study of malaria parasite biology across its entire life cycle through a vertebrate host and a mosquito vector under laboratory conditions. Among the four RMPs originally collected from wild thicket rats in sub-Saharan Central Africa and adapted to laboratory mice, *Plasmodium vinckei* has the largest geographical range and includes the largest number of sub-species, demonstrating its deep genetic diversity. Despite affording the same advantages as other RMP species and additionally displaying a large degree of phenotypic and genotypic diversity, *P. vinckei* has seen limited use in the laboratory. Here, we review the contribution of *P. vinckei* to our understanding of malaria and highlight the areas where it could offer an advantage over other RMP species in future studies.

1. Introduction

Malaria is a deadly tropical disease that has plagued mankind for centuries. The disease caused an estimated 627,000 deaths in 2020, of which 77% were children aged under five years old [1]. It is caused by protozoan blood-borne parasites of the genus *Plasmodium*, and is transmitted by mosquito vectors. Most of the disease burden is concentrated in sub-Saharan African countries, where the most virulent species, *Plasmodium falciparum,* is endemic. Progress in reducing the burden of the disease globally has levelled off despite decades of worldwide efforts [1]. The emergence and spread of partial resistance to artemisinin, the first-line anti-malarial drug used in most endemic countries, initially in South-east Asia [2–4] and most recently in Africa [5,6] has raised further concern that we are losing the battle against malaria. Basic research on the biology of *Plasmodium* remains vital, if we are to identify new drugs or develop new vaccines against the disease.

One of the most valuable tools for the study of *Plasmodium* biology are the rodent malaria parasites (RMPs). Between 1948 and 1974, several RMPs were isolated from wild thicket rats and mosquitoes in the forests of sub-Saharan Africa [7] and were then adapted to laboratory mice [8] and mosquitoes [9] (Fig. 1). These species allow access to all stages of the parasite's entire life cycle across a rodent host and a mosquito vector. They have proved indispensable for studying parasite biology and host-parasite-vector interactions.

Four species of RMPs have been identified to date - *Plasmodium berghei, Plasmodium yoelii, Plasmodium chabaudi* and *Plasmodium vinckei.* Of these, the first three species have been put to extensive use as model systems to study various aspects of the disease. *Plasmodium berghei* has been developed into a tractable system for reverse genetics [10] which has enabled the generation and phenotyping of transgenic knockout lines for almost half of the genes in the malaria genome [11,12]. *Plasmodium yoelii* has been mainly used to investigate mosquito stage and pre-erythrocytic liver-stage *Plasmodium* biology, which are much harder to do with the human malaria parasites [13,14]. Several well-characterised cloned lines of *P. chabaudi* are available and have been used for genetic crossing experiments to study drug resistance, host immunity and immunopathology [15–18]. A detailed record of the immense contribution of these RMP models to malaria research can be found in reviews elsewhere [19–22].

The fourth RMP species, *Plasmodium vinckei*, has been largely ignored. *Plasmodium vinckei* parasites have been isolated from more locations in in sub-Saharan Africa than any of the other species [7] (Fig. 1). Eighteen of these isolates were studied by Richard Carter, David Walliker and colleagues at the University of Edinburgh and found to harbour significant genetic diversity compared to the other RMP species [23,24]. The *P. vinckei* parasites, however, have not been used as widely

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as the other RMPs and only a handful of studies have utilised this extensive resource. Phenotype data [22], reference genomes [25–27] and genetic manipulation protocols [28–31] were generated early on for the other three RMP species, thus enabling their adoption in laboratory experiments, whereas these resources have been made available for *P. vinckei* only recently [32]. Here, we review the unique properties of *P. vinckei*, and consider its contribution (however limited) to our understanding of malaria biology and discuss potential uses the model could be put to in the future.

2. Origin of *Plasmodium vinckei*: The anomaly in Katanga to the isoenzymes in Edinburgh

The origin story of *Plasmodium vinckei* is worth retelling since it touches upon the key events in the fascinating history behind the discovery of rodent malaria parasites and their subsequent adaptation to laboratory use. It began in 1943 with the ground-breaking work of Belgian malariologist Dr. Ignace Vincke, stationed at that time in the province of Katanga in present day Democratic Republic of Congo. Vincke discovered a number of malaria parasite sporozoites in the salivary glands of a subspecies of *Anopheles dureni* mosquitoes captured in a gallery forest that he later established did not feed on humans, primates or livestock. This led him to conclude that the vertebrate host was likely to be the wild rodents endemic to the gallery forest, and so he set off on a two year rat-hunt, capturing wild rodents in the forest and examining their blood for parasites [7]. Finally, in 1948, he discovered blood parasites in an African thicket rat (*Grammomys surdaster*) and upon inoculating white mice with its blood, isolated the first rodent malaria parasite, *P. berghei* [8]. Following this, more parasites were isolated from the region and all proved to be *P. berghei* except for one isolate, collected in 1952 [33]. The parasite was distinct from *P. berghei* in its morphology, course of infection and lack of host cell preference for immature erythrocytes. This anomalous parasite was identified as a new RMP species, and named in honour of its discoverer, *Plasmodium vinckei*.

Subsequent expeditions of a similar nature in various regions in sub-Saharan Africa led to the discovery of several more RMPs. Following their isolation, the next challenge was to classify these parasites at the taxonomic level so that they could be put to meaningful use. While the isolates were easily recognizable as either *berghei*-like or *vinckei*-like [34–36], further taxonomic separation proved difficult, based as it was at the time, on minor differences in morphology, developmental characteristics, and geographical distribution.

A definitive solution to this problem came in 1970 in the form of seminal work on enzyme variation in RMPs undertaken by Richard Carter at the University of Edinburgh [37] (See also Richard Carter's own memoirs, published in this issue). Polymorphisms between isoenzymes cause them to migrate at different speeds under electrophoresis, and this can be used to assess the degree of genetic diversity between strains of parasites. Variations in just four isoenzymes - namely glucose phosphate isomerase, 6-phosphogluconate dehydrogenase, lactate dehydrogenase and NADP-dependent glutamate dehydrogenase - were instrumental in clarifying the species and subspecies grouping among the RMP isolates [24]. Lowland *P. berghei* isolates were recognised as a distinct species, and named *P. yoelii,* separate from the singular *P. berghei* species from the highlands of Katanga [23,37]. Similarly, some of the *P. vinckei* parasites isolated in the Central African Republic (CAR) were redescribed as a separate species, *P. chabaudi* [23]. It is truly remarkable to note that the major conclusions of this first foray into the molecular phylogenetics of malaria parasites still hold true after extensive re-analysis using the DNA-based approaches of the current post-genomics era (Fig. 2).

2.1. One species, five subspecies

Detailed phenotyping and enzyme variation studies have resulted in sub-division of the *P. vinckei* clade into five subspecies [24,38]. The *P. vinckei* isolate from Katanga, DRC, was isolated in sporozoite form from *A. dureni millecampsi* mosquitoes and has never been observed in a naturally infected rodent [33]. Rodent infections were established by injecting these sporozoites into *G. surdaster* (woodland thicket rat) and its complete life cycle was characterised in 1969 making it the nominate subspecies *P. v. vinckei* [39]. A second *P. vinckei* parasite was discovered in Nigeria in 1954 [40], in a shiny thicket rat (*Thamnomys rutilans*, currently named *Grammomys poensis*). This sub-species was characterised in 1975 and designated the subspecies name *P. v. brucechwatti* [41]. Meanwhile *P. vinckei* isolated from the Republic of Congo was found to have a characteristic slow development during the liver stages and was named *P. v. lentum* [42]. Discovery of a fourth subspecies in the CAR, *P. v. petteri*, is linked to that of the RMP species *P. chabaudi*, since these two parasites were often found as mixed infections [43]. They were teased apart by cloning and declared as separate entities after observing distinct variations in their isoenzyme mobility patterns [44].

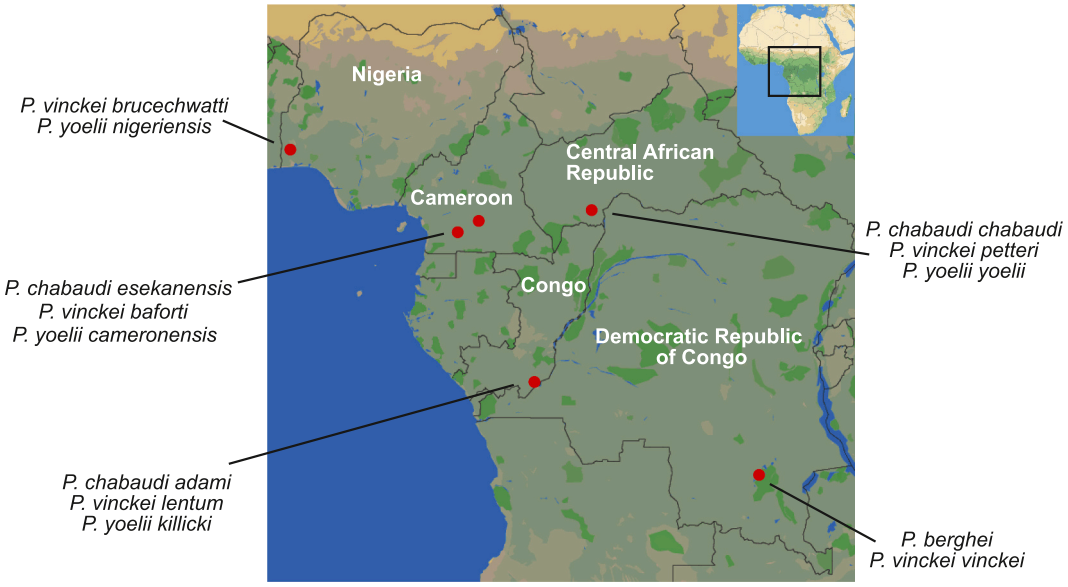

Fig. 1. Rodent malaria parasite species and subspecies and the sites in sub-Saharan Africa where from which they were isolated. *Plasmodium vinckei* is the only RMP species to have been isolated from five different locations. (Modified from [32]).

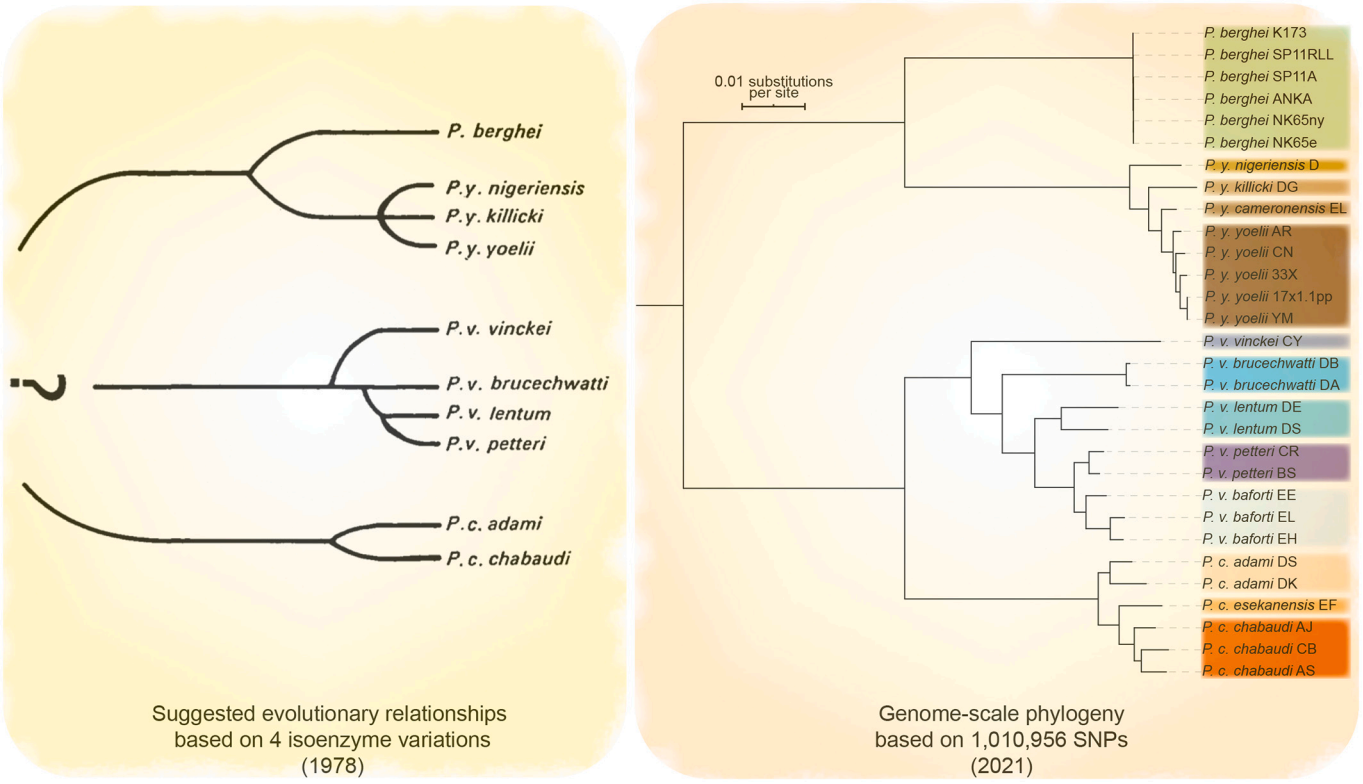

Fig. 2. Evolutionary relationships among rodent malaria parasites. (left) The predicted evolutionary relationships among RMP isolates inferred based on enzyme variations studied by Richard Carter and colleagues (left) [24]. The predictions largely hold true following the production of a highly resolved phylogeny based on single nucleotide polymorphisms in protein-coding genes across RMP genomes (right) [32].

Finally, several isolates collected in Eseka, Cameroon in 1977 [45] were identified as a *P. vinckei* type with isoenzymes distinct from other *P. vinckei* subspecies [38], making them the fifth *P. vinckei* subspecies (recently named *P. v. baforti* [32]). A few more *P. vinckei* isolates have recently been found in Gabon, the mitochondrial sequences of which suggest that they belong to the subspecies *P. v. lentum* [46]. This makes *P. vinckei* the most geographically widespread of the four RMP species with isolates found in all six locations in sub-Saharan Africa where field studies were undertaken (Fig. 1).

2.2. A mixed bag of phenotypes and genotypes

Plasmodium vinckei and *P. chabaudi*, together forming the *vinckei* group of parasites can be readily distinguished from the *berghei* group (*P. berghei* and *P. yoelii*) by their distinct morphological and developmental traits [7]. The *vinckei* parasites establish largely synchronous blood-stage infections, produce an average of 8–10 merozoites per schizont and preferentially invade mature erythrocytes. *Plasmodium vinckei* differs from *P. chabaudi* in that their trophozoites display a characteristic abundance of haemozoin crystals which appear as golden specks in Giemsa's solution -stained parasites. *Plasmodium vinckei* sub-

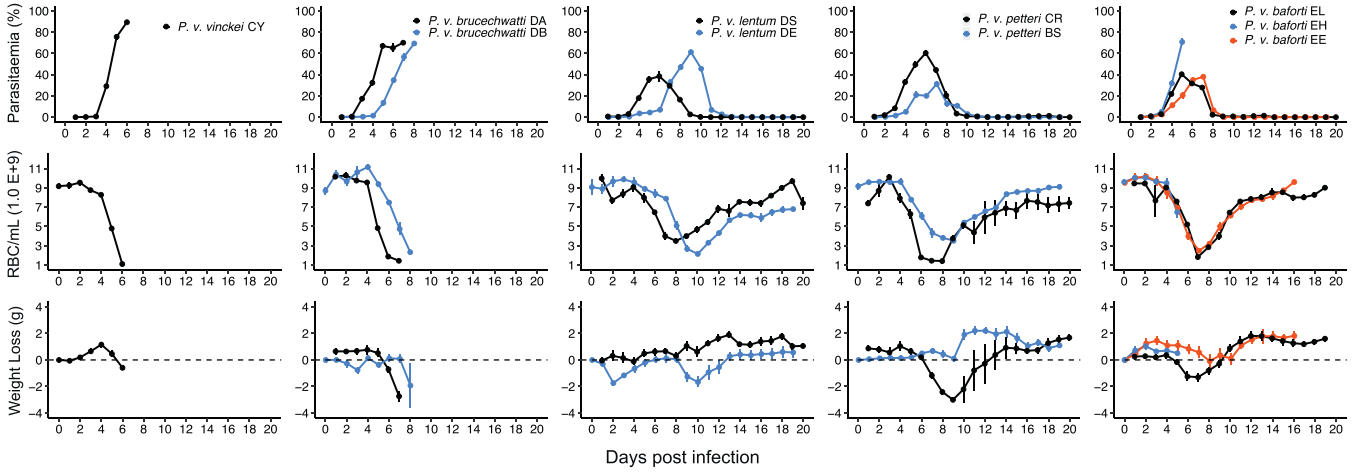

Fig. 3. Infection profiles of ten *Plasmodium vinckei* isolates. Changes in parasitaemia, host RBC density and host weight were recorded during *P. vinckei* infections. Error bars show standard deviation of the mean of five biological replicates. (Reproduced from [32]).

species and strains are morphologically indistinguishable from each other but their course of infection in laboratory mice show extensive diversity (Fig. 3) among the subspecies and between individual isolates [32] (more in **Studying host-parasite interactions in a *P. vinckei* model**). Exoerythrocytic or liver stage schizogony generally lasts for a minimum of 53 h for *P. vinckei* parasites and (exceptionally) for 72 h in the case of *P. v. lentum*. In the mosquito, *P. vinckei* parasites undergo sporogony at an optimal temperature of 24–26 °C to produce mature oocysts with mean diameter of 40–54 μm yielding sporozoites that are around 15–21 μm long. The exception to this is *P. v. vinckei*, a sub-species from the cooler highland forests of Katanga that undergoes sporogony at lower temperatures of 20–21 °C, similar to its sympatric RMP species *P. berghei*.

Early indications of significant genetic diversity within *P. vinckei* came from isoenzyme studies that found only one of the four isoenzymes assayed commonly shared among the *P. vinckei* isolates [24]. This was later confirmed by molecular phylogenies inferred from polymorphisms in the DNA sequences of multiple genes [47,48]. Whole genome sequencing of *P. vinckei* isolates has offered an in-depth view of the level of genetic diversity [32] (Fig. 2). Over a million genome-wide single nucleotide polymorphisms (SNPs) can be found within all RMP isolates sequenced to date. Around half of these polymorphisms exist just within *P. vinckei* in a total of 4,644 core coding genes. The *P. vinckei* subspecies have diverged further from their common putative ancestor than any other RMP species. While *P. v. brucechwatti* and *P. v. lentum* show significant divergences, *P. v. vinckei* is the most diverged of any RMP subspecies with at least five SNPs in the majority of its genes. This level of diversity prompted efforts to produce independent genome assemblies for the five subspecies. In addition to nucleotide-level variations, synteny analysis of these genomes have also revealed large-scale structural variations within *P. vinckei* in the form of chromosomal rearrangements in *P. v. petteri* and *P. v. baforti*. The overall genome size and content of *P. vinckei* genomes are fairly unremarkable compared to other RMPs with the exception of *P. v. vinckei*, which, with a genome size of 18.3 Mb, is the smallest RMP genome sequenced to date.

2.3. Studying drug resistance with a P. vinckei model

Developing parasite lines that show stable resistance to antimalarial drugs is useful in identifying genes involved in drug resistance and the specific genetic mutations that confer resistance [15,49–52]. Historically it has been quite difficult to generate stable drug-resistant lines in *P. falciparum*. It requires prolonged exposure (months or years) of *P. falciparum* culture to increasing doses of drugs [53–55]. Resistant *P. falciparum* lines raised *in vitro* revert back to being chemo-sensitive in the absence of drug pressure raising questions about their fitness in field settings. In contrast, it is relatively easy to obtain stable drug-resistant RMP lines in mice with shorter periods of drug exposure [19]. Growth fitness of these lines can be studied *in vivo* and passaged through mosquitoes, thus allowing for crossing experiments. Lines resistant to a variety of drugs have been developed in *P. berghei*, *P. chabaudi* and *P. yoelii* [19]. A pyremethamine-resistant *P. v. vinckei* line was developed in 1969 after 14 passages through increasing doses of the drug [56]. Similarly, a chloroquine-resistant *P. v. vinckei* line was generated from a pyrimethamine-resistant *P. v. vinckei* background [57]. Contrary to the lethal *P. v. vinckei* parental line, chloroquine-resistant line was non-lethal and showed morphological abnormalities such as reduced haemozoin content and increased vacuolation.

Developing RMP lines with stable resistance to artemisinin had been problematic in *P. berghei* and *P. yoelii* with loss of resistance observed upon withdrawal of drug pressure. However, stable artemisinin-resistant lines have been established in *P. vinckei* [58] and *P. chabaudi* [59]. A *P. v. petteri* strain was shown to develop greater than 12-fold resistance to artether (an ethyl ether derivative of artemisinin) after 44 sequential passages over 700 days in Swiss mice and these parasites were also found to be cross-resistant to quinine and mefloquine [58]. As with the chloroquine-resistant line, the artemisinin-resistant line was non-lethal whereas the sensitive line was lethal, and was able to reach only ~3% peak parasitaemia. Interestingly this was due to a switch in invasion preference to reticulocytes in the resistant line whereas the sensitive line could invade both reticulocytes and normocytes [60]. The *P. vinckei* model has also been used to study parasite recrudescence *in vivo* following artesunate treatment. Dormant ring stages were observed in *P. v. vinckei* infections after drug treatment and were able to reach lethal levels of parasitaemia [61].

2.4. Studying host-parasite interactions in a P. vinckei model

Blood stage virulence is determined by the interplay between host and parasite proteins and is modulated by the host immune response levelled against the parasite. A variety of both parasite and host genetic factors can thus influence the virulence of an infection. Genetic crosses between RMPs with contrasting virulence phenotypes can help identify genes encoding proteins controlling these differences. For example, genetic analysis of cross progeny from fast and slow multiplying *P. yoelii* clones revealed an erythrocyte binding ligand (pyebl) as a determinant of multiplication rate in this species [62,63]. Similarly, *Plasmodium vinckei* lines exhibit a range of virulence phenotypes in laboratory mice [32] (Fig. 3). *Plasmodium v. vinckei* and *P. v. brucechwatti* isolates are highly virulent reaching around 70–90% parasitaemia and killing mice on day 6–8 post infection. Despite both *P. v. brucechwatti* isolates proving lethal to the host, one of the lines takes an exceptionally long time to reach peak parasitaemia. *Plasmodium v. lentum* isolates are not lethal but differ in the rates at which they are cleared by the host's immune system. Both virulent and avirulent isolates have been identified within *P. v. petteri* and *P. v. baforti*.

The course of parasitaemia has also been shown to be host-dependent [64]. *Plasmodium v. vinckei* and *P. v. brucechwatti* isolates, both of which are lethal to laboratory mice, result in low parasitaemia and chronic infections in their natural host, *G. surdaster*. The converse is true in the case of a *P. v. baforti* isolate which is lethal to *G. surdaster* but not to mice. Such stark differences in virulence across both parasite and host lines make for attractive opportunities for the design of experiments aimed at the identification of genetic factors underlying virulence.

Studies exploring host immune response to blood stage infection have mostly gained their insights from the *P. chabaudi* model which shares some similarities in pathogenesis with *P. falciparum* [22]. Immune responses to *P. chabaudi* have been shown to switch from an initial Th1 response to a Th2 response [65]. In contrast, mice infected with the lethal *P. v. vinckei* isolate mount a predominantly Th1 dominated response [66]. Moreover, *P. vinckei* infection is characterised by hypoglycaemia, liver injury and anaemia, similar to severe *P. falciparum* infection in humans [67].

Non-lethal *P. chabaudi* infections have been shown to provide cross–protection against virulent *P. vinckei* strains but not against *P. berghei* [68–70]. Studies with mixed species infections of *P. yoelii* or *P. chabaudi* with *P. vinckei* have shown contrasting results largely influenced by different experimental parameters, with non-lethal *P. vinckei* strains either causing a lethal outcome [71] or reduced mortality [72] when in mixed-species infections.

2.5. Studying chronobiology with P. vinckei

Another field of investigation that has potential use for the *P. vinckei* malaria model is the study of biological rhythms in the malaria parasite. Several malaria species maintain biological rhythms during their asexual replication within the blood, called the intra-erythrocytic developmental cycle or IDC. Depending on the species, each IDC usually lasts for multiples of 24 h (h), at the end of which a synchronous bursting of asexual blood stage schizonts occurs, releasing merozoites into the blood. These cycles of RBC lysis manifest as periodic fevers in the host, a clinical characteristic of the disease. Malaria parasites

intrinsically control their rhythms and entrain their internal clocks to the timing of as yet unknown host cues [73,74] related to host rhythm [75], metabolism [76] or immune responses [77]. Mismatches with the timing of host cues may confer, for example, a growth-related fitness advantage, or potential transmission costs [75,78] whilst rhythm plasticity can also contribute to drug resistance [79,80].

Synchrony differs between the *berghei* and *vinckei* groups of RMPs. *Plasmodium berghei* and *P. yoelii* are typically asynchronous and, even if synchronised infections are set up artificially, the parasites typically lose synchrony within a few cycles. Most studies have used *P. chabaudi* to study parasite chronobiology since it causes a synchronous infection with 24 h periodicity that is timed to the host's circadian rhythm, typically undergoing schizogony at midnight when mice are awake and active. *Plasmodium vinckei* parasites also maintain synchrony, have 24 h IDCs and show periodicity in their gene expression [81] like *P. chabaudi*. However, differences have been noted in dependence on host rhythms. *Plasmodium vinckei petteri* and *P. v. vinckei* always undergo schizogony 24 h from the time of inoculation of ring stages [82,83]. They do not calibrate the timing of their IDC to host daily rhythms like *P. chabaudi*. On the other hand, *P. v. lentum* displays an intermediary phenotype relying both on the time of inoculation and on host rhythms [82]. Comparing *P. vinckei* to *P. chabaudi* may therefore provide further insights into the mechanisms by which the parasite entrains its clock to the timing of host cues.

2.6. Studying RMP evolution with P. vinckei genomes

Correlations between the geographical origin of each RMP species and subspecies and their phenotypic characteristics suggest that the diversification of RMPs was driven by evolutionary forces resulting from diverse host, vector and environmental conditions within isolated ecological niches. For example, *P. berghei* and *P. v. vinckei*, both from the DRC, are distinct from the rest of the RMPs with a lower optimal transmission temperatures and smaller genomes. The former (and possibly the latter) characteristic is most likely due to climactic and host-vector differences in the highland forests of Katanga, shared by both these parasites. Highland forests are at an altitude of 1000–7000 m with mean temperatures of 21 °C whereas the lowland forests lie at an altitude of less than 800 m with a mean temperature of 25 °C. Different host-vector systems are prevalent in the lowland forests (*G. poensis* – unknown mosquito vector) and the highland Katangan forests (*G. surdaster–Anopheles dureni millecampsi* [84]).

Prior to the genome sequencing of *P. vinckei* isolates, the differences between lowland and highland RMPs could not be investigated at a molecular level. *Plasmodium berghei,* isolated from highland forests of Katanga, has no subspecies counterpart from the lowland forests. Inter-species divergence between *P. berghei* and the lowland RMP species *P. chabaudi* and *P. yoelii*, are too wide to make meaningful comparisons between their respective genomes. *Plasmodium vinckei* genome sequences have allowed genome-wide assessment of selection pressure on protein-coding genes in the highland *P. v. vinckei* against the four lowland dwelling subspecies [32]. Genes encoding GAMER (gamete-release protein), PIMMS43 (*Plasmodium* Infection of the Mosquito Midgut Screen 43) and TRAP (thrombospondin-related anonymous protein), proteins that play critical functions in transmission, were found to be under positive selection. Subspeciation among the lowland parasites is associated with positive selection of several exported and rhoptry-associated proteins, 28 kDa ookinete surface protein and a protein phosphatase (PPM8). Further investigation of these genes could provide clues about the molecular mechanisms that drive speciation in malaria parasites. The possibility of inter-subspecies genetic crosses among *P. vinckei* isolates should be explored as linkage analysis of the progeny from such crosses could immensely aid in elucidating these mechanisms.

Another opportunity that *P. vinckei* genomes have afforded is the re-evaluation of the evolutionary patterns of multigene families within the RMPs. Several highly polymorphic multigene families are located in the sub-telomeric chromosomal regions of the parasite genome, encoding a wide variety of exported proteins involved in processes such as immune evasion, cytoadherence, nutrient uptake and membrane synthesis. Various evolutionary pressures have influenced the rapid evolution of these multigene families in the form of copy number variation and rampant sequence shuffling leading to phenotypic plasticity. Phylogenetic analyses of multigene family members typically reveal the evolution of structurally distinct groups of genes forming robust clades with each family thought to have distinct functions [27,32,85]. Multi-species clades formed by orthologous members from different species represent structural orthologs with conserved roles across these species whereas species-specific clades formed by repetitive gene duplications possibly provide an advantage to the propagation of a particular species. Multigene family phylogenies based on *P. berghei, P. yoelii and P. chabaudi* have revealed instances of species-specific expansions but the addition of *P. vinckei* members have allowed us to re-evaluate many of these clades. Many clades thought to be *P. chabaudi*-specific expansions have orthologs in *P. vinckei* and are thus family expansions that have rather occurred in the common *vinckei* group ancestor. Overall, a high level of orthology is observed between *P. chabaudi* and *P. vinckei* genes forming several *vinckei*-group clades, in contrast to more species-specific clades in *P. berghei* and *P. yoelii*. It thus seems that multigene families have evolved quite differently across the *vinckei* and *berghei* groups of RMPs.

2.7. Outro

Plasmodium vinckei has not been as widely used in laboratory studies of malaria as the other RMPs. However, the few studies that have employed this species have uncovered a myriad of interesting phenotypes that are available for further investigation. This should now be possible since their utility as a laboratory malaria parasite system has been greatly enhanced with the generation of new resources. Resources available for the *P. vinckei* model stand thus; reference genomes for all five *P. vinckei* subspecies, genotype and virulence phenotypes for ten isolates, transcriptomes for five isolates (including a 6 h time-series IDC transcriptome for *P. v. vinckei*) and explorable phylogenies of their multigene families (available through PlasmoDB portal [86], https://plasmodb.org/plasmo/app/, and iTOL server [87] https://itol.embl.de/shared/2lCr6w0mdDENs). Genetic manipulation has been demonstrated in *P. v. vinckei*, which could potentially be an exceptional line for this purpose as the combination of high synchronicity and peak parasitaemia results in excellent schizont yields for transfection. Genetic crossing has been demonstrated in one subspecies, albeit with suboptimal results, and requires further optimisation of their transmission through the commonly used laboratory mosquito *Anopheles stephensi*. This should be achievable through careful analysis of the optimal temperature and timing of infectious feeds, and through the repeated passage of the lines through mosquitoes. Thus, the *P. vinckei* parasite lines are a worthy addition to our laboratory toolset of the rodent malaria parasites used to study malaria biology.

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