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Assessing fitness costs in malaria parasites: a comprehensive review and implications for drug resistance management

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Abstract

Artemisinin-based combination therapy (ACT) remains a broadly effective anti-malarial drug combination, but the emergence of resistance is threatening its effectiveness. Limiting the spread of these drug-resistant parasites and delaying the emergence of resistance in new areas are of high priority. Understanding the evolution of resistance relies on discerning the fitness costs and benefits associated with resistance mutations. If the cost associated with resistance in an untreated host is sufficiently large relative to the benefit of resistance in a treated host, then the spread of resistance can be mitigated by ensuring sufficient hosts free from that active pharmaceutical ingredient. There is no straightforward way to measure these fitness costs, and each approach that has been used has its limitations. Here, the evidence of fitness costs as measured using field data, animal models, and in vitro models is reviewed for three of the main current or past first-line treatments for malaria: chloroquine (CQ), sulfadoxine-pyrimethamine (SP), and artemisinin derivatives (ART). Despite the difficulties of assessing fitness costs, there is a good amount of evidence of fitness costs in drug-resistant *Plasmodium falciparum* parasites. The most persuasive evidence comes from resistance reversal observed following the cessation of the use of chloroquine. Comparable evidence cannot be obtained for SP- and ART-resistant parasites, due to the absence of complete cessation of these drugs in the field. Data from in vitro and animal models are variable. While fitness costs are often observed, their presence is not universal across all resistant strains. The extent and nature of these fitness costs can vary greatly depending on the specific genetic factors involved and the ecological context in which the parasites evolve. As a result, it is essential to avoid making broad generalizations about the prevalence or impact of fitness costs in drug-resistant malaria parasites. Focusing on fitness costs as a vulnerability in resistant parasites can guide their evolutionary trajectory towards minimizing their fitness. By accurately predicting these costs, efforts to extend the effectiveness of anti-malarials can be enhanced, limiting resistance evolution and advancing malaria control and elimination goals.

Keywords Malaria, *Plasmodium falciparum*, Fitness costs, In vitro, Rodent model, Resistance management, *Pfcrt*, *Dhfr*, *Dhps*, *kelch13*

Background

Malaria, a life-threatening disease, continues to be a global burden. There are approximately a quarter billion cases annually, with over 600,000 deaths each year [1]. In 2015, the World Health Assembly adopted the Global Technical Strategy for Malaria 2016–2030 (GTS), whose goal is to reduce global malaria case incidence and mortality rates by at least 90% by 2030. Although the global

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death toll has decreased over the last decades, decreases have recently become more stagnant and case numbers are nowhere near eradication goals. Despite valiant efforts to effectively treat malaria, anti-malarials have been met with the development of drug resistance. Currently, artemisinin-based combination therapy (ACT) remains a broadly effective anti-malarial drug combination left, but its efficacy is threatened by the emergence of resistance against artemisinin-derived compounds and their partner drugs across various countries in Southeast Asia [2–4] and more recently in several countries in sub-Saharan Africa [5–9]. Limiting the spread of these mutant parasites and delaying the emergence of resistance in new areas are of critical importance [1]. To develop more effective resistance management strategies than those that are currently being implemented, there is a need to understand the specific forces of evolution acting upon malaria parasites and how clinical and public health interventions interact with these.

The rate at which resistance evolves depends on the fitness costs and benefits associated with resistance mutations [10]. Biological fitness refers to a parasite's ability to survive and reproduce within its current host, while also successfully transmitting to new hosts. Fitness is highly dependent on genotype and environmental interactions, meaning that certain genotypes (e.g., resistant genotypes) may have a high fitness in some environmental conditions (e.g., in the presence/absence of drugs). Resistance management can exploit this by altering the environment in such a way to exploit fitness costs and lower the fitness of resistant parasites. Competition from other parasite strains is one factor that may lower the fitness of resistant parasites [11–14]. The level of such competitive suppression is dependent on the intrinsic fitness of the resistant parasite. It is generally assumed that the mutations that confer resistance could alter the function of a protein in ways that disrupt the parasite's metabolism, bringing about fitness costs. If the cost of having a resistance mutation in an untreated host is greater than the benefit of having the mutation in a treated host, then resistance is unlikely to spread [15]. This observation is the basis for resistance management strategies such as drug rotations, low-dose treatment, or adaptive therapy in anti-malarial, anti-microbial, and anti-cancer therapies [16–20]. These strategies all depend on the wild-type susceptible parasites to outcompete the emerging resistant parasites by either temporarily preventing the use of a drug through its substitution with another drug, or, more experimentally, by using a limited drug dose that is aimed to maintain a population of wild-type parasites that can outcompete the resistant ones [16]. The efficacy of these resistance management strategies is largely dependent on a fitness cost associated with drug resistance.

Fitness costs associated with drug resistance have been reported in a wide range of disease systems. A review by Andersson and Levin [21], found that most resistant bacteria carry a fitness cost, which was confirmed in a meta-analysis published in 2015 [21, 22]. Fitness costs have also been detected in viruses, such as Zika virus [23], influenza [24], and most notably HIV [25].

Reduced replication has additionally been reported in another apicomplexan, *Toxoplasma gondii* [26]. The effects of fitness costs are furthermore commonly found among other organisms, such as reduced growth rates, lower germination rate, smaller roots, and delayed flowering in herbicide-resistant plants [27], and decreased fecundity and survival in insecticide-resistant insects [28, 29]. Finally, drug-resistant cancer cells have been shown to be outcompeted by wild-type cells in the absence of treatment [18, 30, 31]. However, it is important to note that fitness costs are not universally found [32, 33]. Interestingly, the opposite has also been observed, where drug resistance is associated with a fitness benefit even in the absence of treatment, such as observed for *Leishmania* parasites resistant to pentavalent antimonies [34]. This review summarizes the latest literature on the evidence of fitness costs associated with drug resistance in *P. falciparum* parasites. The last specific reviews on this topic were published by Walliker et al. in 2005 [35] and Rosenthal et al. in 2013 [36]. Since then, artemisinin resistance has spread on a large scale, and a large number of new studies have been published. This work will summarize the current state of the field, evaluate the evidence supporting the presence or absence of fitness costs, and highlight the current knowledge gaps.

How are fitness costs measured?

Measuring fitness costs associated with drug resistance is not a trivial undertaking. First, one needs to define fitness. In bacterial systems, this is often measured as growth rate or peak pathogen density [37], and such methods are frequently used to measure fitness in malaria studies as well (e.g., [12, 36]). However, malaria parasites have a more complex life cycle than bacterial species. Gametocytes, the sexual stages taken up by the mosquito, are the main determinants of a parasite's fitness since they determine transmission. However, these are difficult to measure due to low densities and frequent absence in in vitro cultures. Therefore, asexual parasite growth is typically used as a surrogate for fitness. Additionally, malaria parasites have two hosts: the vertebrate host and the mosquito vector, and fitness in both hosts determines a parasite's overall fitness, but fitness in mosquitoes is rarely studied. Even in the human host, it is impossible to directly observe fitness costs of a parasite, as it requires measuring fitness in the absence of treatment, which for

obvious ethical reasons cannot be performed in patients. Therefore, these assays are frequently done in an experimental animal or in vitro models, and these have their specific limitations. Lastly, fitness costs may be exacerbated by the presence of other genotypes. Competitive growth assays are important for the overall determination of fitness, as is being done for other organisms such as HIV, bacteria and fungi (reviewed in [14, 25, 38–41]).

Although there is no standard technique for measuring fitness costs, there are three main categories where evidence is typically coming from: field observations, animal models, and in vitro assays (Fig. 1). Genetic surveillance field studies, most notably the reversal from mutant to wild-type alleles following either a temporal discontinuation (such as seasonal drug use) or complete discontinuation of a drug, can be used as an indication of fitness costs. While these studies do not directly measure fitness costs, they are indicative that the relative fitness of a drug-resistant strain is lower than that of the susceptible strain when resistant allele frequency decreases over time. The problem with this approach is that (1) fitness cost can only be measured after resistant parasites have reached high enough frequencies to detect a reduction, (2) drugs are rarely fully removed from the market, and (3) once resistance is fixed in the population, resistance may persist due to absence of wild-type parasites to replace them. The combination of both animal and in vitro models can fill this gap. In animal models, it is possible to measure all aspects of fitness throughout the life cycle of the parasites. However, animal models are mostly limited to species of malaria parasites that do not infect humans, typically rodent and avian malaria parasites, and extrapolations need therefore be made with caution. For instance, the host immune response is likely to play a role in competitive interactions between parasites, and although animal models include a functioning immune system, the functional organization of the immune system differs significantly between humans and experimental animal species [42]. However, despite differences, there are a multitude of overlapping features,

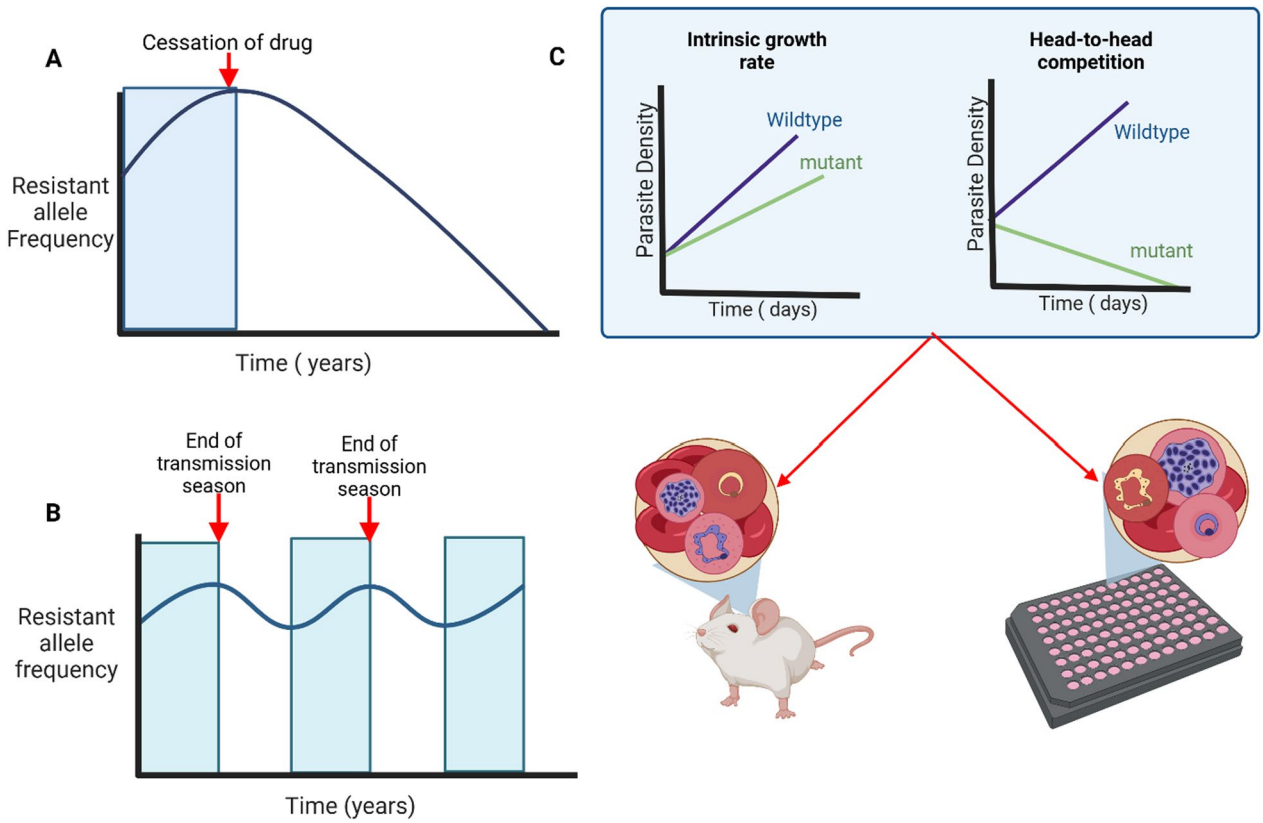

Fig. 1 Different approaches to analyzing the occurrence fitness costs. **A** Analysing the frequency of resistance alleles in the field prior to (shaded) and following the cessation of the focal drug (unshaded area). In the occurrence of a fitness cost, the frequency of the resistant allele is expected to decrease in the population in the absence of drug use in the population. **B** Analysing the frequency of resistance alleles in the field across transmission seasons. The reduction of resistant allele frequency during periods of low-level drug use on a population level (unshaded areas) are indicative of fitness costs. **C** Measuring growth rates of mutant and wild-type strains in animal models and in vitro culture. These studies could compare intrinsic growth rates as well as examine head-to-head competition. Created with BioRender.com

and both rodent malaria and avian malaria models have generated a wealth of knowledge.

In vitro studies fill another gap by allowing a direct measurement of fitness costs of the *P. falciparum* parasite itself. The limitation of an in vitro system is that it only gives an approximation of the conditions within a human host. Within the human host, malaria parasites are subject to multiple environmental shifts, such as fluctuations in temperature, oxygen tension, nutritional and immune status, which are normally either absent or very stable within in vitro cultures [43]. Additionally, malaria parasites find themselves in a large diversity of ecologically different environments (Fig. 2). Typical in vitro assays focus on the erythrocytic life cycle of *P. falciparum* and thus ignore the migration through the dermis and liver stage within the human host [44]. If fitness costs are being expressed in different parts of the life cycle, or in different organs, it will not be detected in current in vitro models. Mosquito assays could be followed up by in vitro culturing, but typically this is performed with a limited set of strains that consistently produce gametocytes in the artificial environment of an in vitro system. Current developments of 3D multi-cell-type liver organoids [45] could, in the future, allow for the study of potential fitness costs during the liver stage of the life cycle. A final limitation of in vitro work is that it is often limited to a few laboratory strains with a risk of confounding genetic backgrounds with resistance traits. Recent advances in genome editing strategies may fill this gap though. Crispr-cas9 technology allows now for the creation of isolines and isolating the impact of single mutations on fitness and competitive ability [46]. However, laboratory strains, whether they be genetically engineered or strains that have been in the laboratory long enough to no longer resemble field strains, are likely to differ from strains that are in naturally occurring infections. Measuring fitness costs lacks a straightforward approach, with each method presenting its own set of limitations.

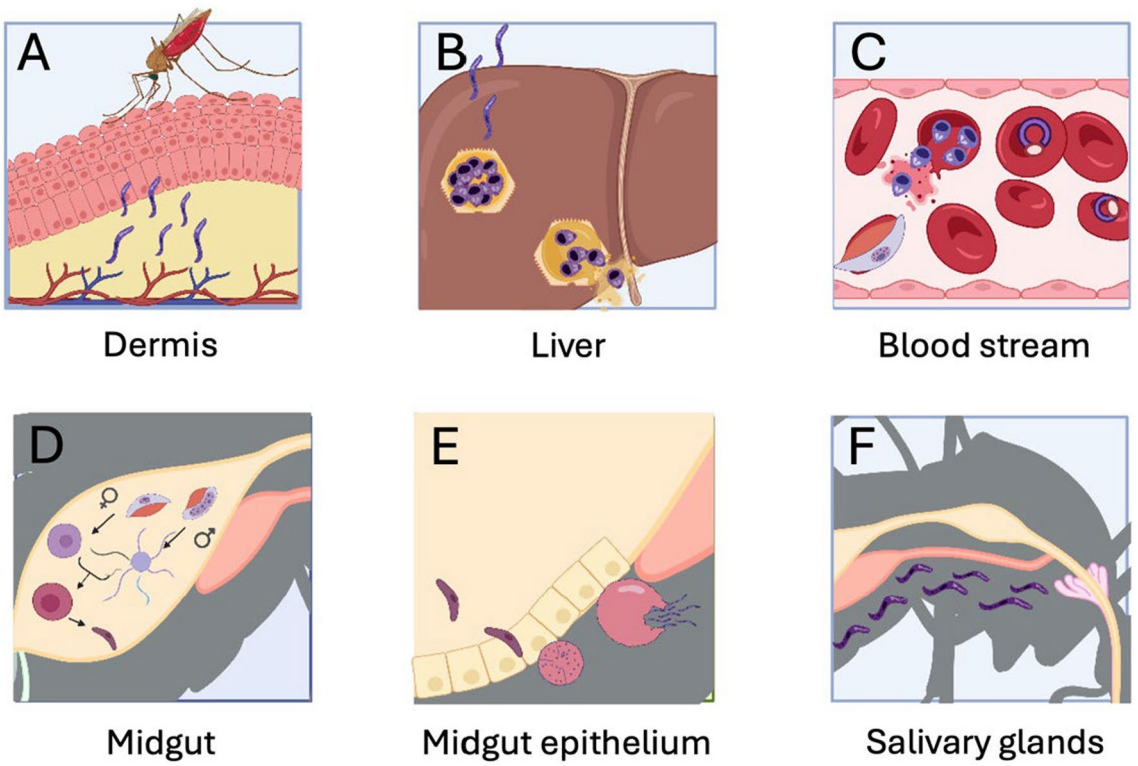

Fig. 2 The different ecological environments and life stages of *P.falciparum* where fitness costs could arise. **A** To enter the bloodstream, sporozoites must cross the endothelial barriers of the dermis and epidermis. Variations in their gliding motility and ability to evade skin-resident immune cells may affect parasite fitness [125]. **B** Upon reaching the liver, sporozoites enter hepatocytes, where they undergo asexual replication. Parasites may vary in replication rate, capacity to evade liver-specific defenses, host cell manipulation, and exploitation of hepatocyte resources [125]. **C** Rapid asexual reproduction occurs in the bloodstream. Here, parasites may compete for resource utilization, invasion efficiency, immune evasion, and gametocyte production. [217, 218]. **D** Environmental conditions like temperature and pH vary significantly in the mosquito midgut, impacting parasite fitness in terms of survival, gamete formation, and zygote development [184, 216]. **E** Ookinetes must penetrate the midgut epithelium, successfully form oocysts, and replicate. Parasites may differ in their success at these stages, as well as in host resource extraction and immune evasion within the mosquito [184]. **F** In the mosquito haemolymph, sporozoites rely on motility to reach the salivary glands, with fitness differences arising from variations in motility, immune evasion, energy storage, and effective energy use [219]. Created with BioRender.com

A comprehensive understanding can be constructed through the combination of evidence from various perspectives. Here, the evidence for fitness costs in these three forms (field, animal models, and in vitro models) is reviewed for three of the primary first-line treatments historically used for malaria: chloroquine (CQ), sulfadoxine-pyrimethamine (SP), and artemisinin derivatives (ART).

Evidence for fitness costs in chloroquine-resistant parasites

Chloroquine (CQ) was one of the earliest drugs that was used globally for malaria treatment, starting in the middle of the twentieth century. Unfortunately, starting in the 1990s, CQ was increasingly replaced as a first-line treatment in numerous countries due to the emergence and global spread of CQ-resistant (CQ-R) parasites [47, 48]. Malaria parasites need to detoxify heme, which is a toxic byproduct of hemoglobin digestion. Chloroquine targets the detoxification pathway, resulting in the parasite being poisoned by its own waste products [49]. Resistance to chloroquine is primarily mediated by mutations in the chloroquine resistance transporter gene (*pfcrt*), though mutations in a second recognized drug transporter, *P. falciparum* multidrug-resistance 1 (*pfmdr1,* specifically N86Y), is also associated with decreased sensitivity to CQ [50]. The mutations on *pfcrt* confer the ability to efflux chloroquine from the intracellular digestive vacuole, which is the site of action for chloroquine [49]. The resistance transporter is pivotal for parasite growth and replication by maintaining the osmotic homeostasis of the digestive vacuole [51]. Since *pfcrt* plays a crucial role in the survival of the parasite, it is likely that mutations present in *pfcrt* could interfere with its physiological function, thereby reducing its overall fitness.

Field evidence of fitness costs of CQ-R

Infections with CQ-R parasites have been found to have lower parasite densities than CQ-S infections, suggestive of a fitness cost [52, 53]. In contrast, gametocyte carriage has been found to be higher in resistant infections, and resistant gametocytes to be more infectious to mosquitoes compared to wild-type infections in the absence of treatment [53–55]. While these findings are conflicting, the best evidence for the cost of resistance in CQ-R parasites comes from the increase in the frequency of chloroquine-sensitive (CQ-S) parasites following the removal of the use of CQ for malaria treatment. A progressive decline in in vitro and in vivo resistance over the course of the 1980s on the island of Hainan in China after the suspended use of chloroquine in 1979 was the first of such evidence [56, 57]. Malawi, the first country to replace CQ with SP as first-line treatment in Africa in 1993, was the first to report a full reversal of susceptibility by 2001 [58, 59]. Since then, a similar reversal to almost complete elimination of the resistant *pfcrt K76T* following CQ discontinuation has been reported in a few places, such as in Zambia [60], Tanzania [61], and Mozambique [62]. Additionally, a significant reduction of this mutation has been seen throughout sub-Saharan Africa [63–68]. Similarly, the frequency of resistant parasites has been found to decrease during the low transmission season. Drug use is expected to be low during these times, and a slight reversal of resistance during these time periods additionally suggests that resistant parasites have a lower fitness than susceptible parasites [15, 52, 69].

The speed with which frequencies decrease following cessation of CQ use differs drastically by region. Much slower drops in *pfcrt*76T frequencies have been reported in lower transmission areas such as South America and Southeast Asia [12, 70, 71]. Field studies in South America and Asia have also observed that the fitness cost of parasites harboring the SVMNT haplotype in positions 72–76 (7G8 allele) may be less severe than that of parasites carrying the CVIET haplotype (Dd2 allele) [72]. Petersen et al. [73] also observed a lower fitness cost with the 7G8 allele compared to Dd2 allele in their in vitro studies. Another main driving factor of the speed of resistance reversal is the efficiency with which new drug policies are implemented. Despite an official policy change, chloroquine use was still ongoing in some areas due to continued availability in the public and private sector, prescription for treatment of *Plasmodium vivax* infections, or chemoprophylaxis in children with sickle cell disease. Such practices could have presumably led to a lower drop in resistant mutants [63, 74, 75]. Also, the use of piperaquine or amodiaquine, which are structurally similar to chloroquine, could lead to continued selective pressure for resistance [72, 74, 76–78]. Additionally, the replacement drug artemether-lumefantrine could also speed reversal by selecting wild-type parasites [79–81]. Finally, the speed of reversal is also dependent on the level of competition between parasite strains.

Resistance on the China–Myanmar border is hypothesized to persist due to a lack of competition from other strains [82]. Since the speed of reversal is dependent on so many different factors, most importantly the continued use of chloroquine, which is extremely difficult to quantify, CQ-R frequency is problematic to use for quantifying fitness costs. It is also important to note that the focus on *pfcrt76T* might give an incomplete picture. For instance, in French Guiana, 76T remained fixed in the population, but susceptibility returned through the *pfcrt* C350R mutation [83]. Conversely, in Madagascar, while the wild-type pfcrt allele was prevalent, chloroquine treatment failure was linked to the *pfmdr* N86Y mutation [84].

Animal model evidence of fitness costs of CQ-R

The first study of CQ-R fitness costs involved competition assays between CQ-R *Plasmodium chabaudi* parasites with wild-type parasites in mice in the 1970s. Surprisingly, the CQ-R parasites outcompeted sensitive forms in all experiments, even when resistant parasites started at a much lower frequency than sensitive parasites [85]. Such higher fitness of resistant parasites could have been the result of the selection process for resistance which required several passages through mice and mosquitoes under increasing drug pressure. This selection process could have inadvertently selected additional adaptive mutations [35]. More recently, genetic tools have allowed for the engineering of specific mutations that circumvent the confounding factor of drug selection. The introduction of V2721F and V2752F mutations in the UBP-1 gene in *Plasmodium berghei* via CRISPR-Cas9 led to parasites with decreased artemisinin and chloroquine resistance but also reduced growth rate. The resistant parasites were easily outcompeted by susceptible parasites and having both mutations appeared to be lethal [86]. Similarly, an edited N331I mutation in the *P. berghei* chloroquine resistance transporter gene led to reduced susceptibility to the structurally similar piperaquine, but also to reduced growth rates [87]. With the advancement of genetic tools, it is anticipated that the number of studies on CQ-R in animal models will increase, providing deeper insights into its associated fitness costs.

In vitro model evidence of fitness costs of CQ-R

CQ-R parasites co-cultured in vitro with CQ-S parasites were outcompeted by the susceptible parasites, leading to a reversion to nearly fixed susceptible populations over the course of 70 days. This was found for various strains originating from Asia or South America [88]. The study demonstrated that this fitness cost was most likely the result of the disruption of haemoglobin catabolism. When parasites were grown in a media lacking essential amino acids that are found in haemoglobin, the CQ-R parasites were significantly more impaired in their growth than the CQ-S parasites. Using gene editing, a notable fitness cost was demonstrated to be associated with several mutations in the *pfcrt* gene [12]. However, the magnitude of the cost was dependent on the genetic background, with parasite strains GB4 and Cam783, the two predominant mutant lineages found in Africa, having a smaller fitness cost than Dd2 [12]. Another study found that several genetically modified parasites, engineered to express geographically diverse *pfcrt* haplotypes, also had reduced growth rates [73]. However, this study also reported on a strain isolated from Cambodia, Cam734, which had evolved a moderate degree of chloroquine resistance, but did not carry a detectable fitness cost. Cam734 carried a complex set of *pfcrt* mutations that could explain this observation [73]. A similar finding of reduced growth rates associated with *pfcrt* mutations engineered in different genetic backgrounds also confirmed that the genetic background played an important role. The 7G8 genetic background, commonly found in South America, was mildly protective against fitness reductions [89]. Parasites engineered to contain the *pfcrt* F145I mutation, which leads to resistance to the structurally similar drug piperaquine, were fully outcompeted within 12 days by wild-type parasites and demonstrated enlarged, translucent digestive vacuoles [90]. It is important to note that while studies utilizing genetic modifications provide valuable proof of concept regarding potential fitness costs, these findings may not accurately represent the fitness costs observed in naturally occurring malaria parasites. Beyond *pfcrt*, fitness costs have also been associated with mutations in the *pfmdr1* gene in transfected laboratory strains, where mutations at the 1034, 1042, and 1246 codons led to significant reductions in parasite growth in vitro. This growth deficiency was found to be linked to a reduced viability of merozoites [91]. Finally, a recent study revealed the role that the previously overlooked *P. falciparum* amino acid transporter (*pfaat1* gene) is likely to play an important role in CQ resistance evolution. Gene editing revealed that the *pfaat1* S258L mutation increased chloroquine resistance but was associated with a fitness cost. In contrast, the *pfaat1* F313S variant reduced resistance while compensating for the fitness cost [92]. Reduced growth rates are not always associated with resistance. For instance, when comparing common lab strains HB3 (CQ-S) with Dd2 (CQ-R), the resistant strain had higher proliferation rates [93] and was able to outcompete the susceptible strain in head-to-head competition [94].

Evidence for fitness costs in SP-resistant parasites

SP is an anti-malarial drug that was introduced as a first-line treatment in the 1960s. In contrast to CQ-R strains, which emerged two decades after its introduction, resistance to SP was reported soon after its introduction [95–97]. SP resistance was first observed on the Thai-Cambodian border in the mid-1960s, and became a significant challenge within a few years of its introduction [98]. In vitro SP resistance was demonstrated in South America in the 1980s [99]. By 1995, many South American countries replaced CQ with SP as first line treatment, but SP resistance had already been documented and it quickly spread due to subsequent drug pressures [100]. In Africa, sensitivity to SP started declining in the late 1980's [98]. Due to a rise in drug resistance, SP has officially been removed as the first-line anti-malarial treatment in most of sub-Saharan Africa and replaced with

other drugs in the early 2000s [101, 102]. SP interrupts parasite folate synthesis by targeting dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR). Folate biosynthesis is essential for pyrimidine production and, in turn, for the parasite's DNA replication [103]. The mutations in *P. falciparum* that confer resistance to SP occur in the gene dihydrofolate reductase (*pfdhfr*), with polymorphisms N51I, C59R, S108N, and I164L, and in dihydropteroate synthase (*pfdhps*) occurring with S436A, A437G, K540E, A581G, and A613S/T [104, 105]. These mutations could impact the efficiency of the enzyme. Since resistance to SP is imparted by an intricate combination of these mutations, it is challenging to assess the effect of individual mutations in these genes on parasite fitness [106].

Field evidence of fitness costs of SP-R

Studies reporting on the changes in resistance markers following the cessation of SP as first-line treatment often reveal contradicting results that differ by location, time since cessation of SP use, the extent of reduction in SP pressure, and the particular single nucleotide polymorphisms (SNPs) or haplotypes under selection. Despite SP not being recommended as first-line treatment for well over a decade in most of Africa, the prevalence of mutant alleles in SP resistance markers is still very high throughout the region. However, the particular combination of *pfdhfr*/*pfdhps* mutations that are reported varies (reviewed in [107]). In general, the frequency of both *pfdhps* double mutants (A437G/K540E) and *pfdhfr* triple mutants (N51I/C59R/S108N) appeared to be increasing over time in the African region at least up to 2009 [108]. These so-called quintuple mutations in *P. falciparum* have been associated with SP clinical failure [109]. In Uganda and Rwanda, these quintuple mutants remained high in frequency even seven to eight years after cessation of SP as first-line treatment. However, *pfdhfr* I164L and *pfdhps* A581G mutations, associated with high levels of resistance, dropped in frequency [110, 111]. In Cambodia, no wild-type haplotypes were observed in the population even after more than 10 years of discontinuation of SP [112]. Stably high frequencies of mutant haplotypes following discontinuation of SP have also been reported in Bioko Island [113], Gabon [114], Venezuela [115], and Thailand [116, 117]. In Kenya and Malawi, the frequency of mutant alleles in SP resistance markers continued to increase despite official cessation of the drug as recommended first-line treatment [118, 119]. Interestingly, *pfdhps* A581G emerged in Kenya after the official policy change [120]. In contrast though, in Ethiopia, triple *pfdhfr* mutants decreased in the first few years after cessation of SP, with *pfdhps* double mutants staying the same [121]. Whereas in southern Mozambique, a drop in *pfdhps* double haplotypes was observed several years following cessation of SP use, meanwhile in neighboring KwaZulu-Natal province in South Africa, *pfdhfr* triple mutants stayed the same [122]. Finally, in Peru, a significant drop of *pfdhps* triple mutations (437G/540E/581G) and *pfdhfr* triple mutations (51I/108N/164L) were observed several years following cessation of SP [123].

It is difficult to derive any conclusions from these observations. The use of SP in intermittent preventive therapy during pregnancy (IPTp), in infants (IPTi), and as seasonal malaria chemoprevention (SMC) is still recommended. Furthermore, artesunate-SP is still on the list of recommended artemisinin-based combinations by the World Health Organization (WHO) [124]. Therefore, there is continued selective pressure for resistance against SP, and this pressure likely differs across different regions. For instance, the population age distribution could impact continued pressure, where areas with an expanding population sees a higher proportion of [124] pregnancies and thus drug pressure via IPTp (Intermittent preventive treatment of malaria during pregnancy) [125]. Due to its use as chemoprevention, SP is readily available in many areas which can further increase the use of SP as treatment. Furthermore, drugs with similar modes of action are currently used to treat other diseases in malaria-endemic areas, such as co-trimoxazole for HIV-infected individuals and for acute respiratory infections in children [124, 126, 127]. Cross-resistance between co-trimoxazole and SP is a reasonable concern in areas where SP is still used [108, 128], however, studies have shown that the use of co-trimoxazole is not associated with an increase in SP mutants and can, in fact, prevent malarial infections [101, 129, 130].

Drug pressure can also vary locally due to dissimilarities in immune responses, transmission levels, and cultural practices [122]. Geographical differences, particularly in drug use, could lead to differences in resistance frequency, such as seen between villages and the capital in Sudan [131]. In areas where mutants are fixed in a population, reductions in mutant frequencies are less likely to be observed despite the reduction of drug pressure. This is a consequence of there not being any wild-type mutants left to compete with the drug-resistant parasites in the absence of treatment unless wild-type parasites are reintroduced or reverse point-mutations take place [115].For instance, studies of field isolates collected from Cambodia and Venezuela have reported that no wild-type *pfdhps/pfdhfr* sequences were detected [112, 115, 132]. Lastly, it is also important to note that SP has a long half-life somewhere between 100 and 230 h [133], in contrast to a half-life of less than one hour for artesunate [134], which leads to prolonged selective pressure on any infecting parasite. When comparing the parasite density

of infections containing SP-resistant mutants versus wild-type infections, a weak association of lower parasite count has been found for 108N and 51I mutants compared to wild-type parasites from a small study in Sudan [53]. However, reduced parasite numbers have not been observed in other studies [128, 135, 136]. Interestingly, SP-resistant parasites have repeatedly been found to have both higher gametocyte carriage and higher infectivity to mosquitoes compared to sensitive parasites, which could potentially explain their sustained high frequencies [53, 122, 131, 137, 138]. Overall, no evidence of strong fitness costs was observed based on changes in resistance markers in the field. However, considering there is significant ongoing exposure of parasites to SP, this absence of evidence is not proof for the absence of fitness costs associated with SP resistance mutations.

Animal model evidence fitness costs of SP-R

Due to the complex combinations of mutations in both *pfdhfr* and *pfdhps* that confer resistance to SP in field isolates, in vivo animal model experiments on the fitness of SP-resistant parasites are absent as it is difficult to assess the cost associated with a specific mutation [106]. However, in vivo experiments that elucidate the fitness costs of resistance to either pyrimethamine or sulfadoxine have been reported, but the results are either not consistent or not sufficient evidence to support the presence of a fitness cost. Yamauchi et al. [106] evaluated the fitness of sulfadoxine-resistant transgenic *P. berghei* parasites within mice with mutation A394G (corresponding to *pfdhps A437G*) by comparing the growth rate of the sulfadoxine-resistant clone to that of susceptible ones in the absence of sulfadoxine treatment, either in single infections or in competition with each other. They found no significant difference in parasite growth rate, gametocyte production, or mosquito infections between the resistant and wild-type clones. Two studies assessed the fitness costs of pyrimethamine-resistant (Pyr-R) parasites in mice. Shinondo and colleagues [139] investigated whether Pyr-R carried a fitness cost by comparing two lines (Pyr-R and Pyr-S) in terms of parasite replication rate and the ability to produce gametocytes. They found that both lines had similar asexual growth rates, gametocyte conversion rates, and exflagellation rates. Contrastingly, Rosario et al. [85] previously observed Pyr-S lines of *P. chabaudi* to outgrow the Pyr-R line in two out of the three occasions, but the sample size was low. As far as is known, these are the only animal model studies that have compared the fitness of either pyrimethamine- or sulfadoxine-resistant parasites with wild-type parasites. With current genetic tools, it would be easy to establish these differences in isogenic lines that differ in the various mutations in *dhfr* and *dhps* linked to resistance to SP, similarly as conducted by Yamauchi et al. [106].

In vitro model evidence fitness costs of SP-R

Several types of studies have been conducted to establish the fitness costs associated with the most common mutations of *pfdhfr* and *pfdhps*. When taking mixed genotype isolates from the field and placing them in culture, two studies showed a loss of resistant genotypes over time in a small number of isolates. However, in both cases, sample sizes were so small that these results could have been obtained by chance [140, 141]. Enzyme efficiency studies show conflicting results. Sandefur et al. [142] compared the kinetic properties of wild-type alleles and three mutant alleles and found that mutant enzymes were, surprisingly, more efficient in vitro than the wild-type form. However, Sirawaraporn et al. [143], in contrast, showed that triple and quadruple mutant enzymes were critically jeopardized in vitro compared to the wild-type enzyme. However, enzyme efficiency towards dihydrofolate does not necessarily need to impact organismal fitness [144]. Experiments using a transgenic system to transform the bacteria *Escherichia coli* or the yeast *Saccharomyces cerevisiae* to carry alleles of the *dhfr* gene show modest to major costs associated with the high level of antifolate resistance [145, 146]. The addition of 164L mutation led to a substantial fitness cost [147]. Overall, fitness landscape studies show that once a parasite is a highly resistant mutant, they could be trapped on a peak in the fitness landscape. Even if the fitness of a wild-type parasite in a no-drug environment is highest, reverse mutation to this ancestral state may be inaccessible as intermediate mutants would have detrimental fitness costs [144, 148]. Hence, the reversal of resistance in these cases is most likely through migration and the spread of ancestral strains. Finally, competition assays between *dhfr* mutant and wild-type parasites in *T. gondii* did not show any difference in growth rates in vitro, but there were minor differences in fitness when tested in mice [149]. Interestingly, they found a strong fitness cost for *dhfr* F223S mutants combined with C59R, which may explain why the former mutation has not been commonly observed in the field.

Evidence for fitness costs in artemisinin-resistant parasites

Currently, there are six artemisinin-based combinations available and recommended by the WHO for uncomplicated falciparum malaria: artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-SP, dihydroartemisinin-piperaquine, and artesunate-pyronaridine [124]. ACT became a common, widespread therapy treatment in the early 2000s [35]. Artemisinins and its derivatives (ARTs) are highly effective and quickly

terminate several life cycles of intraerythrocytic *P. falciparum* parasites, but are always partnered with a long-acting drug that clears residual parasites to shorten the regimen, ensure an efficacious treatment, and to safeguard ARTs from drug resistance evolution [13]. The exact mode of action of ARTs is unclear, but is thought to be through the activation of free heme leading to the formation of reactive oxygen species. Carbon-centred free radicals then damage essential parasite proteins and activate the endoplasmic reticulum (ER) stress response, leading to a lethal buildup of polyubiquitinated damaged proteins [150, 151]. Despite the use of artemisinin-based drugs for several decades, high level resistance has still not been reported, demonstrating the difficulty of parasites to evolve resistance against this drug. The first case of reduced efficiency of artemisinin was reported from Cambodia in 2008 [3, 152], which was associated with C580Y mutations in the Kelch (*Pfk13*) propeller domain [153, 154]. Subsequently, these mutations have spread across southeast Asia [155]. Additionally, cases of C580Y resistance haplotypes were reported in Guyana in 2015 [156], as well as other *pfk13* mutations associated with resistance in several sites in Africa more recently [157–159]. The molecular mechanism of the observed reduced sensitivity is still largely unresolved. It has been proposed that *pfk13* plays a key role in presenting damaged proteins for ubiquitination (needed for maintaining cell homeostasis), eventually leading to cell death. Mutations in *pfk13* could lead to a defect in presenting damaged proteins for polyubiquitination, which could contribute to the survival of the parasite [150]. Additionally, *pfk13* mutations could also be responsible for the increased export of ART-damaged proteins (reviewed in [160]). The question is whether such altered pathways associated with *pfk13*, or any other, unknown mutations outside this region, could impact the fitness of parasites.

Field evidence fitness costs of ART-R

Despite over two decades of intense use of artemisinin, there is still no widespread resistance, which is longer than any other anti-malarial. A potential explanation for this observation is that any high-level resistance adaptation is associated with an inhibitory large fitness cost. However, alternative explanations are the usage of combination therapy, its short half-life and thus shortened pressure, improved drug use practices, or simply physiological constraints to the adaptation. Since ACT is still used as first-line therapy, there is no molecular surveillance data on the changes in the frequency of artemisinin mutations associated with resistance in the absence of the drug. Seasonal fluctuations in drug use could give an insight into the presence of fitness costs associated with *pfk13* mutations, similar to what is observed for CQ-R parasites, however, based on available information, such studies have not yet been published. Additionally, no study has reported an association between *pfk13* mutations and multiplicity of infection. However, considering that *pfk13* mutants are predominantly circulating in low transmission areas, it is unlikely to find associations in these areas as complex infections are generally low in these areas [161–163]. The more recent appearances of *pfk13* in higher transmission areas on the African continent may allow us to address this outstanding question. One study compared *pfk13* mutations and gametocytaemia but did not find an association between the two [164]. It is noteworthy that the presence of the partner drug will always confound field observations, and assessing the fitness cost of ART-associated mutations in isolation will be difficult. Therefore, animal models and in vitro assays are more likely to provide clear insight in the extent of a fitness cost associated with *pfk13* mutations.

Animal model evidence fitness costs of ART-R

Several artemisinin- or artesunate-resistant parasites have been experimentally selected in rodent malaria models. However, fitness differences between the resistant lines and their progenitor strains were not assessed in all of these resistant strains, and most strains did not have mutations in the K13-propeller domain. In a recent study, Zheng and colleagues [165] selected artemisinin- and piperaquine-resistant *P. berghei* strains and identified nine mutations in the *P. berghei* K13-propeller domain with associated prolonged parasite clearance, though none of these mutations are commonly found in the field in *P. falciparum*. These strains were mildly resistant to artemisinin, but curiously, resistance reversed in these experiments despite continuous drug pressure. The introduction of common *K13* homologs in *P. berghei* using gene editing showed some mutations having quite severe growth defects (the homologs of *P. falciparum* M476I and R539T), while no growth defects were associated with F446I and Y493H. Interestingly, they were unable to successfully introduce the equivalent mutations of C580Y and I543T, which could suggest these mutations were associated with severe deleterious effects [86]. A study of *Aotus* monkey infections of genetic crosses between C580Y mutant and C580Y wild-type parasites also hints at a potential fitness cost associated with C580Y in terms of reduced ability to recrudesce. However, statistical power in this study was too low to make this a firm conclusion [166]. Finally, studies of ART-resistant strains with mutations not associated with the K13-propeller domain did find fitness costs in terms of slower growth [167] and rapid reversal following cessation of drug exposure [168]. Such a slower growth rate could in and of itself lead to protection of the killing effect of artemisinin

due to its short half-life [169]. However, other studies using slow-clearing *P. chabaudi* or *P. berghei* parasites did not report slower growth rates in the absence of treatment [170–172].

In vitro model evidence fitness costs of ART-R

A series of independent in vitro competitive growth assays of genetically engineered strains have been performed to determine the impact of *pfk*13 mutations on the fitness and competitive ability of mutant parasites. These have all shown competitive suppression of C580Y mutants [46], though the genetic background plays an important role in the magnitude of the fitness cost [13, 173]. R539T mutants were also competitively suppressed [13, 173] or had a reduced growth rate in the absence of competition [12]. In a series of head-to-head competitions of genetically distinct strains, it was also found that strains with C580Y mutations were the poorer competitors [14]. Overall, it appears that C580Y may have a larger fitness cost than other *pfk13* mutations, depending on their genetic background. This may be surprising considering C580Y is the most widespread mutation in Southeast Asia, though it could potentially explain its near absence in Africa, where most malaria infections occur [158, 159]. Other artemisinin-resistant strains were also shown to have lower growth rates [174], or be outcompeted by their isogenic parental susceptible strain, leading to a reversal of resistance [175–177]. Finally, a yeast model of artemisinin resistance further confirmed that resistant yeast cells had severe growth defects [178].

Evidence in other drugs

Fitness costs have been observed for other drugs. In one of the first in vitro head-to-head competition experiments, *P. falciparum* parasites with mutations in cytochrome b (leading to atovaquone resistance) were outcompeted by the wild-type parasites [179]. Additionally, *P. falciparum* parasites with increased copies of the putative drug transporter *pfmdr1* (leading to mefloquine resistance) were outcompeted by parasites with single *pfmdr1* copy numbers, leading to a reversal in about 60 days of culturing [180]. In co-cultures of wild-type and genetically engineered mutants *pfmdr* N86Y and Y184F, pleiotropic interactions were found between the different mutations regarding drug susceptibility and fitness costs. Most interestingly, the 86N wild-type polymorphism, which is associated with decreased sensitivity to lumefantrine, incurred competitive fitness costs, but only in the presence of the 184F mutation [50]. Finally, a piperaquine-resistant *P. berghei* strain was found to have a more than 80% reduction in growth rate compared to their isogenic wild-type strain, while no cost was associated with a lumefantrine-resistant strain [181].

Evidence for fitness costs in mosquitoes

Studies that focus on the fitness costs of *Plasmodium* parasites within the mosquito vector are scarce [182]. Nevertheless, the mosquito plays a vital role in the transmission of *Plasmodium* spp. to humans and the passage through a mosquito is an essential step in their life cycle. While in the mosquito, parasites undergo fertilization, transformation to ookinetes, and development of sporozoites [183] (Fig. 2). A potential fitness cost could be expressed at any one of these phases. Malaria parasites experience a substantial reduction in numbers as they develop inside the mosquito vector. During the midgut stage, the parasite must persist for over 20 h outside a protective host cell. Without a protective host cell, the parasites have to defend themselves against components of the human immune system (that came along with the blood meal), the natural mosquito midgut microbial flora, and the innate immune system of the mosquito [184, 185]. With a decreased population size, resulting population bottleneck, and a novel and hostile environment, a fitness cost expressed during this life stage is likely. Moreover, a fitness cost during this transmission stage is likely to have a large impact on resistance evolution [186]. On the other hand, it has been demonstrated that parasite diversity is greater in mosquitoes than in humans, suggesting mosquitoes could be a reservoir for parasite strains [187, 188], though this is not always found [189]. If a higher diversity can be maintained in mosquitoes, this suggests resistant parasites could experience an ecological space of reduced competition. However, empirical data on fitness costs during mosquito infection are scarce [184].

A large fitness cost during the mosquito transmission stage has been observed for atovaquone-resistant parasites. Goodman et al. [190] tested three atovaquone-resistant strains of *P. berghei* for transmissibility and found that *cytB* mutants tested were unable to transmit from mouse to mouse via *Anopheles stephensi* mosquitoes. Additionally, they found that the *P. falciparum cytB* M133I and V259L mutants, when grown in culture, were severely impaired in their mosquito infectivity than their parental lines and had a reduced number of oocysts when infection did occur. Similarly, in an avian malaria model, a fitness cost was demonstrated for artesunate-resistant *Plasmodium relictum* parasites. Here, the artesunate-selected lines generated lower oocyte and sporozoite burden in their natural mosquito host, *Culex quinquefasciatus,* in comparison to the ancestral line [182]. However, clear fitness costs are not always demonstrated. Mutant *pfcrt*76T from human volunteers had an equal number of sporozoites produced as wild-type parasites [187]. Using transgenic *P. berghei Pbdhps-A394G* mutants with the equivalent mutation of *pfdhps* A437G, no differences were observed in growth in mice, in gametocyte

production, nor in number of oocysts produced [106]. Another study found that pyrimethamine-resistant *P. berghei* parasites were found to have the same growth rate and gametocyte production in mice, the same exflagellation rate, ookinete rate, and oocyst production in mosquitoes. However, a slower sporozoite production in the resistant strain compared to the sensitive strain was observed, which could reduce fitness in the field [139].

Fitness costs during the mosquito infection stage may also be observed in the field. In *An. arabiensis* mosquitoes collected in southern Zambia, the wild-type *pfcrt* K76 polymorphism was twice as often observed in the mosquito abdomen than in human populations and five times as frequent in the mosquito salivary glands, suggesting a cost for the mutant in the mosquito stage [191]. A similar finding was made for pyrimethamine-resistant parasites in mosquitoes in Zambia [192]. However, such clear patterns are not always found. In a household comparison of humans and mosquitoes, most resistance markers had similar prevalence in humans and mosquitoes, except for *pfmdr86Y,* which was more prevalent in mosquitoes [193]. Similarly, in Equatorial Guinea, no differences were observed between human and mosquito samples from the same household for either *pfcrt*, *pfdhfr,* or *pfdhps* mutations [189]. In contrast, both in Kenya and Uganda, a trend towards marginally increased prevalence of mutant *pfcrt* 76 T was observed in mosquitoes [194]. Thus, overall, there is no clear pattern of fitness costs associated with resistance in mosquito vectors, but the evidence is thin.

Compensatory mutations

There may be mutations that, either fully or partially, restore the fitness of resistant mutants. This would result from natural selection for the fittest parasite under such sustained drug environments [73]. These so-called compensatory mutations are a distinct form of epistasis in which the new mutation has an advantageous effect on the organism's fitness when a deleterious mutation is present but would otherwise be neutral or disadvantageous on its own [195]. Such compensatory mutations are common and have been found in a variety of organisms, such as antibiotic-resistant bacteria [196–198], insecticide-resistant insects [199], antiviral-resistant HIV viruses [200–202], and drug-resistant *T. gondii* parasites [26].

This review's assessments of fitness cost, as observed from field evidence, are, therefore, likely the end product of both the cost and any evolved compensatory mechanisms. For malaria parasites, compensatory mutations have been frequently suggested, particularly in the case of SP resistance which requires a series of multiple point mutations. Indeed, using transfections in *S. cerevisiae*, it was uncovered that resistance reached high levels upon the first fixation of the S108N mutation, but the growth rate was reduced. The growth rate was partially restored upon the successful insertion of three other point mutations [144]. A relationship between SP resistance alleles and the upregulation of the guanosine triphosphate cyclohydrolase-1 (Pfgch-1) protein has also been suggested to have a compensatory function [117, 203]. In the case of *pfcrt* mutations, it is still unclear which are compensatory and which ones play key roles in mediating resistance to chloroquine. Various mutations within the *pfcrt* gene have been suggested to have predominantly a compensatory role [73, 89], and a study on the protein's function using *Xenopus laevis* oocytes as a model system showed that mutations are required to be obtained in a specific order to avoid loss of fitness [204]. Beyond mutations in the *pfcrt* gene, multiple genome-wide mutations have been identified that may restore the fitness cost of *pfcrt* mutations [205]. For artemisinin resistance, it has been shown that the genetic background or off-target mutations are important for the successful spread and fixation of *k13* resistance mutations [173]. Several compensatory mechanisms have been proposed. An upregulation of nutrient permeable channels was found that may compensate for amino acid shortage found in artemisinin-resistant parasites [206]. Additionally, inserting two or three asparagines after the *k13* region in mutant haplotypes could play a compensatory role [207]. Lastly, a mutation in the *PfAtg18* gene that promotes more efficient nutrient acquisition through an autophagy-like pathway may be involved in restoring fitness costs [208].

Understanding the occurrence and mechanisms of compensatory mutations is critical for developing resistance management strategies. These mutations could prevent resistance reversal by keeping parasites in a state of intermediate fitness. Peaks in the fitness landscape represent optimal fitness, while valleys represent lower fitness; parasites may become trapped on a smaller peak, unable to evolve toward a higher peak without crossing a valley of reduced fitness [148]. However, new mutations that arise after the cessation of a drug could circumvent this fitness valley. In French Guiana, a novel *pfcrt* C350R substitution was found that rapidly spread and restored CQ susceptibility. However, this mutation was also shown to be involved in piperaquine resistance, which likely contributed to its spread [83]. Novel genetic tools combined with model systems, such as *S. cerevisiae* [144], *X. laevis* oocytes [204], and a high-mutator *P. berghei* strain [87] have the potential to lead to a more detailed identification of compensatory mechanisms associated with various resistance mutations and, thus, a better understanding of the constraints that resistant parasites face during resistance reversal evolution.

Fitness costs are not a fixed parameter

This review discusses studies that aim to elucidate the magnitude of a fitness cost. However, it is important to note that fitness costs are unlikely fixed parameters [11]. Malarial infections frequently consist of mixed-genotype strains, and the resulting within-host interactions impact the growth and, thus, the fitness of each co-infecting strain. Consequently, fitness costs could be amplified by competition with other parasite strains. Indeed, in a study using the same strain of rodent malaria *P. chabaudi,* it was shown that the fitness cost could range anywhere from 0 to 100%, depending on whether these costs were observed in competition or during singular infection and which metric of fitness was used, e.g. asexual growth rate or gametocyte production [11]. In an area of high transmission, such as many areas in sub-Saharan Africa, individuals with malaria are often infected with multiple variants, with some cases having as many as 14 different strains. Conversely, in areas of low transmission, such as many areas in Southeast Asia, individuals may be infected with as few as a single strain [161–163, 209, 210]. A fitness cost may, therefore, be greater in high transmission areas compared to low transmission areas, which likely contributes to the observation that resistance has historically emerged most frequently in Southeast Asia rather than on the African continent [36, 211]. However, beyond differences in within-host competition, fitness costs likely depend on a range of other environmental factors within the human host, such as immune response, co-infections, anaemia, nutritional status (e.g. [174, 212, 213]), as well as differences within the mosquito host, such as species, immune response, microbiome, co-infections, and climate factors [214–216]. As such, both animal models and in vitro models are unlikely to capture field-relevant fitness costs, and the best estimates are obtained from field observations, which, as discussed above, have their limitations as well. While these studies are nevertheless invaluable, it is important to note that the extrapolation of fitness costs, such as using a single fixed parameter in a mathematical model, is unlikely to provide accurate estimates of resistance evolution.

Concluding remarks

Fitness costs associated with drug resistance have been reported in a wide range of disease systems. Attempts to provide evidence of fitness costs associated with drug resistance in *P. falciparum* parasites is a laborious undertaking, due to the parasite's complex life cycle and shifting between hosts (vertebrate host and mosquito vector). Despite these difficulties, there is a great amount of evidence of fitness costs in drug-resistant *P. falciparum* parasites. The most persuasive evidence comes from resistance reversal observed following the cessation of the use of chloroquine. It is possible that *pfcrt* is intrinsically associated with a greater cost in fitness than other common drug resistance mechanisms. However, it is equally plausible that similar costs exist for SP- and ART-resistant parasites, but these parasites have not been exposed to a drug-free environment like CQ-R parasites, where such patterns might become evident. Data from in vitro and animal models are overall suggestive of the presence of a fitness cost. However, these costs were not always found for both CQ and the other drugs, and fitness costs are likely dependent on the specific locus and mutation and the genetic background of the parasites. Therefore, sweeping conclusions and assumptions on the presence of a fitness cost as a general phenomenon cannot and should not be made.

Yet, being able to predict the magnitude of fitness costs better or construct the microenvironment of parasites to have maximum fitness costs is an important step toward improving resistance management. It would allow for the control of the evolution of resistance, thus extending useful lifespans of drugs. It would also predict areas of higher likelihood of resistance emergence and aid drug discovery towards resistance-retardant drugs. Fitness costs can be harnessed in novel resistance management strategies. For anti-malarial resistance management, low-dose treatment has been shown in *P. chabaudi* to reduce drug resistance evolution effectively, but also hinges on the presence of a fitness difference between resistant and susceptible parasites [16]. Knowing the magnitude of this cost allows for a prediction of the efficacy of these resistance management strategies but also predicts the speed of resistance evolution in the opposite scenario, when drugs are used at a large scale, such as during mass drug administration. Ultimately, these fitness costs may be used to force resistant parasites down an evolutionary path with the greatest possible fitness cost. For instance, if chloroquine was reintroduced in combination therapy—not for clinical benefit, but to exert selective pressure on parasites to carry *pfcrt* mutations and incur a fitness cost—this strategy could compliment other resistance management strategies, provided that cross-reactivity and/or drug toxicity do not pose limitations. By leveraging fitness costs as the Achilles' heel of resistant parasites, their evolutionary trajectory can be steered towards maximal fitness impairment. Such endeavours, guided by meticulous predictions of fitness costs, hold the key to prolonging drug efficacy, curtailing resistance spread, and ultimately advancing the goal of malaria control and eradication.

Abbreviations

ACT Artemisinin-based combination therapy
CQ Chloroquine

SP	Sulfadoxine-pyrimethamine
ART	Artemisinin derivatives
WHO	World Health Organization
GTS	Global Technical Strategy for Malaria
CQ-R	Chloroquine resistant
CQ-S	Chloroquine susceptible
SP-R	Sulfadoxine-pyrimethamine resistant
SP-S	Sulfadoxine-pyrimethamine susceptible
SNPs	Single nucleotide polymorphisms
IPTp	Intermittent therapy during pregnancy
IPTi	Intermittent therapy in infants
SMC	Seasonal malaria chemoprevention
Pyr-R	Pyrimethamine resistant
Pyr-S	Pyrimethamine susceptible
ER	Endplasmic recticulum
ART-R	Artemisinin derivative resistant
ART-S	Artemisinin derivative susceptible

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SH conceived the idea for the project. All authors were involved in performing the literature search. XS and BS wrote the first draft of the manuscript. SH supervised the overall work. All authors critically revised and approved the final manuscript.

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