Why is the effect of malaria parasites on mosquito survival still unresolved?

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Despite almost a century of effort, the question of whether malaria parasites kill their mosquito vectors remains open. Some direct comparisons of the longevity of infected and uninfected mosquitoes have found malaria-induced mortality, whereas others have not. Here, we use meta-analysis to show that, overall, malaria parasites do reduce mosquito survival. However, mortality effects are more likely to be detected in unnatural vector–parasite combinations and in studies of longer duration. Until these factors are systematically investigated, no firm generalities are possible.

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During the two weeks that malaria parasites (*Plasmodium* spp.) take to complete development in the mosquito, they can cause substantial damage (Box 1). However, conventional wisdom postulates that natural selection will favour parasites that do not influence vector survival because a parasite that kills its vector will kill itself. Although this view of virulence evolution has been rejected on theoretical grounds (selection maximizes fitness not life expectancy [1,2]), it still pervades the malaria literature [3–6]. The evidence for it is contradictory. Some indirect field data support the idea [7] whereas others do not [8]. Direct laboratory comparisons of the survival of malaria-infected and uninfected mosquitoes have also produced conflicting results, with some reporting reduced mosquito survival [9–17] and others finding vector survival to be unaffected [18–30] (Table 1). This inconsistency is surprising: if *Plasmodium* does impose a direct cost on mosquito survival, why is it not consistently found in controlled laboratory experiments? Understanding the reason for this variation is crucial not only to answer the general question of whether malaria parasites are detrimental to their vectors, but also to identify the conditions under which vector survival could limit the epidemiology and evolution of malaria.

We conducted an analysis of 22 previously published laboratory studies (Table 1) to determine the overall direction and magnitude of *Plasmodium* effects on mosquito survival, and to test whether the variation in outcomes could be explained by experimental design. We used quantitative meta-analytical methods [31,32], which require that summary statistics and sample sizes are given for each study so that a standard measure of statistical effect size can be computed. This information was missing from several of our studies [10,12,18–21,23–26,29]. Consequently, meta-analysis was conducted on only 11 of the original 22 studies (Table 2). In total, these 11 studies provided information on 24 separate experiments.

Overall effect

The proportion of studies that have found a statistically significant detrimental effect of *Plasmodium* is similar to the proportion that have not (41% and 59%, respectively; Table 1). For all accounts of reduced survival to have arisen by chance alone (Type 1 errors), there would need to be ~360 unpublished studies with null results, in addition to about nine showing increased survival. Given the experimental effort involved in survival studies and the novelty of showing that malaria is a longevity enhancer, this degree of under-reporting seems unlikely. The lack of any studies showing a significant positive influence of *Plasmodium* is interesting because, if parasites can manipulate mosquito longevity, they might be expected to enhance it (at least during the time when oocysts are growing [33]) – by, for instance, reducing fecundity and hence the longevity costs of reproduction [34]. The absence of this effect suggests that no such manipulation is occurring.

Quantitative analysis shows that, in 22 out of 24 experiments, *Plasmodium*-infected mosquitoes had poorer survival than their uninfected counterparts (Table 3) and that overall, malaria does reduce mosquito survival. The mean effect size was similar when experiments were treated as independent units and when analysis was conducted on the average effect size of all experiments in a study. Of the 24 experiments that we examined, there was no relationship between effect size and sample size ($P = 0.26$, $n = 24$).

What factors influence experimental outcome?

To include as many data as possible, all further meta-analyses were conducted at the level of individual experiments.

Choice of species

Of the 22 studies that we identified, ten were studies of natural combinations of vector and parasite species, ten were of unnatural combinations and, for two, the natural vector is unknown (Table 1). Of the ten studies that used an unnatural vector–parasite combination, seven found that infection decreased mosquito survival; none of the ten studies that used natural associations reported a significant effect, although all studies exhibited a tendency towards poorer survival in the infected group (Table 2). The magnitude of *Plasmodium* effects on mosquito survival is substantially greater when novel pairings are used (Table 3, $P = 0.04$, $n = 22$).

This analysis supports the notion that *Plasmodium* is harmful only in novel vector species, an idea that is often proposed to explain the lack of

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Box 1. Mechanisms by which *Plasmodium* can damage its vector

Tissue damage
Mosquito midguts are perforated when ookinetes pass through them [a]. In addition to the physical damage, this perforation might increase susceptibility to bacterial infection or invasion by other parasites during subsequent feeding [b,c].

Physiological disruption
Levels of aminopeptidase, a digestive enzyme, are reduced in mosquitoes with oocysts [d].

Resource depletion
Infected mosquitoes have lower concentrations of amino acids in their haemolymph than those that are uninfected, and their midguts use eight times as much glucose [e,f].

Cost of immunity
Mosquitoes can mount a diverse array of immune responses when invaded by pathogens [g]. *Plasmodium* infection elicits the transcriptional activation of at least six different immune markers in the human malaria vector *Anopheles gambiae*, particularly when parasites are invading the midgut and salivary glands [h,i]. The production of these molecules could be energetically costly and divert resources away from growth and maintenance. Some mosquitoes kill oocysts by melanotic encapsulation, a process that is known to reduce mosquito ovary size and protein content when directed against filarial worms [j]. Mosquitoes selected to be refractory to *Plasmodium* have also been shown to have poorer fitness than susceptible mosquitoes in the absence of infection [k].

Behavioural modification
Infected mosquitoes have less salivary apyrase (a platelet inhibitor) [l]. Consequently, these mosquitoes spend more time feeding, probe more often [m,n], are more persistent in biting [o] and feed more often [p] than uninfected mosquitoes. These changes in behaviour are likely to increase the risk of infected mosquitoes being detected and killed while feeding.

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virulence in studies of natural infections [27,30]. Assuming that the unnatural combinations used in the laboratory are a random sample of novel pairings, is there an *a priori* reason to expect them to be more virulent? It is often assumed that only maladapted parasites are virulent, so that virulence is high in host–parasite associations that have not co-evolved. Yet neither theory nor empirical studies support this: both increased and decreased virulence can arise from novel host–parasite pairings [35–37]. Certainly, there are many accounts of increased virulence in novel associations [38,39], but ancient virulent associations are common and avirulent interactions are less likely to be noticed [36].

Although a reduction in mosquito survival is more commonly reported in unnatural *Plasmodium*–vector combinations, there are examples of novel combinations that did not result in virulence [26]. Furthermore, a range of outcomes can be found for the same vector–parasite combination, with different studies reporting different effects (e.g. *Plasmodium berghei* in *Anopheles stephensi* reduced survival [12] or did not [26]). Comparative studies, in which the effect of malaria parasites is assessed simultaneously in natural and novel vectors in the same laboratory, are crucial to determine the relevance of co-evolutionary history to mosquito–malaria interactions. We know of no such studies.

Length of study

Mosquitoes cannot transmit malaria until approximately two weeks after infection, when the parasite has transformed into a sporozoite and invaded their salivary glands. Natural selection should minimize virulence, at least until sporozoites have developed. Once sporozoites have developed, natural selection will favour parasites that can increase the biting rate of their vectors, possibly at the expense of longevity [33,40]. This prediction has received empirical support from the observation that sporozoite-infected mosquitoes are more persistent feeders and have greater feeding-associated mortality than their uninfected counterparts [41,42].

Even though only one of the studies in our meta-analysis allowed host biting after initial infection [16], longevity effects were more likely to be detected in

Table 1. Studies investigating the effects of *Plasmodium* on vector survival[a]

Parasite	Vector	In the wild?[b]	Study length (days)[c]	Evidence[d]	Statistics reported?[e]	Control type[f]	Refs
Vector survival reduced							
Plasmodium praecox	*Culex fatigans*	?	5	E	N*	U	[9]
Plasmodium rhadinurum	*Aedes aegypti*	N	3	E	N	U	[10]
	C. fatigans	N	3	E	N	U	[10]
Plasmodium cathermerium	*Culex pipiens*	?	20	E	N*	U	[11]
Plasmodium berghei	*Anopheles stephensi*	N	14	E	–	U	[12]
Plasmodium cynomolgi	*Anopheles dirus*	N	65	E	Y	U	[13]
P. cynomolgi	*A. dirus*	N	65	E	Y	U	[14]
Plasmodium yoelii	*A. stephensi*	N	6	E	Y	U	[15]
P. yoelii	*A. stephensi*	N	6–18	E	Y	U	[16]
Plasmodium chabaudi	*A. stephensi*	N	35	E	Y	U	[17]
Vector survival unaffected							
Plasmodium vivax	*Anopheles punctipennis*	Y	–	O	N	–	[18]
Plasmodium falciparum	various *Anopheles* spp.	Y	–	O	N	–	[19]
P. vivax	*A. punctipennis*	Y	17	O	N	–	[20]
P. falciparum	*Anopheles crucians*	Y	17	O	N	–	[20]
P. vivax	*Anopheles maculipennis*	Y	–	O	N	–	[21]
P. vivax	*A. maculipennis*	Y	7–19	E	N*	U	[22]
P. vivax	*Anopheles quadrimaculatus*	Y	–	O	N	–	[23]
Plasmodium gallinaceum	*Aedes aegypti*	N	7	E	N	U	[24]
P. gallinaceum	*A. aegypti*	N	8	E	N	U	[25]
P. berghei	*A. stephensi*	N	20	E	N	U	[26]
P. falciparum	*Anopheles funestus*	Y	30	E	Y	S	[27]
	Anopheles gambiae	Y	30	E	Y	S	[27]
P. falciparum	*A. gambiae*	Y	5	E	Y	U	[28]
P. falciparum, *P. vivax*	*Anopheles tessellatus*	Y	14	E	N	U	[29]
P. falciparum	*A. gambiae*	Y	5	E	Y	S	[30]

[a]All studies involve experimental blood feeds except Refs [27,30], which used wild-caught mosquitoes.
[b]? indicates that the natural vector is unknown; Y indicates that the species combination is known to exist in the wild (according to Ref. [54]) and N indicates that it is not.
[c]– indicates that the study length is unclear from the relevant paper.
[d]This column indicates whether the conclusions were based on anecdotal observation (O) or experimental testing (E).
[e]– indicates that effect size was not obtainable from reported statistics; Y indicates that statistical analysis was given; N indicates studies without statistical analysis and N* indicates that no statistical analysis was given, but that raw data were given; in these cases, we did an appropriate test.
[f]S indicates that the control group was mosquitoes without sporozoites; U indicates that the control was mosquitoes fed uninfected blood and – indicates that no control was used.

studies that lasted until the sporozoite stage or longer. Effect size was positively related to study length ($P = 0.001$), with the overall effect size being greater for studies ending after sporozoite invasion than for studies that ended before sporozoite invasion (Table 3). These time-dependent effects could arise because sporozoites cause more physiological disruption than oocyst or ookinetes, because parasites alter mosquito behaviour (coincidentally increasing mortality), or simply because small differences in daily survival are more easy to detect over long time periods. Regardless of the mechanism, this analysis strongly suggests that virulence will be underestimated by studies that end before the completion of *Plasmodium*'s extrinsic incubation period.

Dose effects

Malaria biologists have long argued that *Plasmodium* is harmful to mosquitoes only when parasite load is very high [15,21]. This idea has been used to dismiss the possibility that parasites could limit vector populations because few infected mosquitoes carry more than one to two oocysts in the wild [27]. However, the absence of high oocyst burdens could also be due to the mortality of heavily infected mosquitoes [7]. In the laboratory, variable effects of parasite dose have been reported. In one study, mortality increased with the density of asexual parasites, but only at one of two temperatures [12]. In other studies, mortality was unrelated to gametocytaemia [11,17], and oocyst burdens have been correlated with mortality in some [14,16] but not all [17] studies. We know of no studies comparing sporozoite load with mosquito survival.

It is difficult to assess the importance of parasite dose to effect size in our sample of experiments because estimates of blood-stage and sporozoite densities were mostly absent. Some analysis was possible for oocyst burden, a parameter that was reported in five studies (12 experiments) [13–17]. In this sample, there was no relationship between effect size and mean oocyst burden ($P = 0.76$, $n = 12$). Clearly, the data are far from definitive, but they do suggest that oocyst burden is not a large, universal predictor of harm.

Environmental conditions

Mosquito longevity varies with temperature [9,12], season [21], diet [43] and larval density [44,45].

Table 2. Studies used in the meta-analysis of the effects of *Plasmodium* on vector survival[a]

r	*n*	Oocyst load	Mean temperature (°C)	Mean humidity (%)	Refs
0.187	99	–	23	90	[9]
0.387	2692	–	25	83	[11]
0.686	720	–	26	67	[13]
0.195	280	<10	26	67	[14]
0.705	360	10–40			
0.827	480	41–70			
0.907	320	>71			
0.259	207	>75	27	80	[15]
0.056	101	>75	27	80	[16]
0.048	93	>75			
0.032	116	4.4			
0.137	100	4.4			
0.286	100	4.4			
0.010	1527	17.2	27	70	[17]
0.058	1814	44.6			
–0.048	60	–	24	75	[22]
–0.001	100				
0.028	40				
0.060	50				
0.449	60				
0	1128	–	25.3	65.5	[27]
0	1221				
0.005	1600	–	28	80	[28]
0.047	967	2.4	24.5	80	[30]

[a]The Pearson correlation coefficient (*r*) represents the standardized effect size of *Plasmodium* on mosquito survival. We assigned a positive value to the effect size if the survival of uninfected mosquitoes was higher than that of infected mosquitoes. Effect sizes were obtained by converting the one-tailed *p* value for the test of differences in survival between infected and uninfected mosquitoes into a standard normal deviate (*z*) and dividing by the square root of the sample size [55]. For studies that consisted of several different experiments, separate *r* values were computed for each. Mixed effects analysis was used to evaluate the relationship between effect size and experimental design parameters. *n* is the total number of mosquitoes used in each experiment. Oocyst load is the mean number of oocysts on the midgut of an infected mosquito.

Table 3. Mean effect sizes of *Plasmodium* on mosquito survival[a]

Sample	Mean *r*	95% confidence interval of *r*	Number of experiments
All experiments	0.287	0.136–0.470	24
Studies (experiments pooled within a study)	0.259	0.102–0.447	11
Experiments that used natural associations	0.061	–0.004–0.170	9
Experiments that used unnatural associations	0.436	0.201–0.705	13
Experiments that ended before sporozoite invasion	0.129	0.055–0.218	10
Experiments that ended after sporozoite invasion	0.395	0.147–0.664	14

[a]If there was no effect of malaria infection on longevity, effect size would be indistinguishable from zero. Positive effect sizes indicate that mortality is increased by infection. Confidence intervals were obtained by bootstrapping. Statistical analysis was conducted on *Z*-transformed values of the Pearson correlation coefficient (*r*), which represents the standardized effect size of *Plasmodium* on mosquito survival. Results were calculated using the program METAWIN (Sinauer Associates) [55].

The particular rearing, climatic and nutrient regimes used in different labs could therefore influence survival. Furthermore, this variation is likely to influence the ability of mosquitoes to tolerate parasitism. Several studies have shown that *Plasmodium*-induced mortality varies with environmental conditions such as temperature [9,12], diet [17], adult density [11] and bacterial infection [26]. The survival studies that we examined were performed over a wide range of temperatures (21–30°C) and humidities (57–90%). Across experiments, there was no obvious relationship between temperature and effect size ($P = 0.42$, $n = 24$), but there was for humidity ($P = 0.04$, $n = 24$), with less *Plasmodium*-induced mortality at higher humidities. Experiments are required to assess whether this association has any causal basis.

Diet is another factor that could influence mosquito response to *Plasmodium* but, because there was little replication of dietary conditions between studies, we did not conduct any quantitative analysis of this variable. Quantitative analysis was also not possible for larval density because this trait was mentioned in only five studies (and the effect size was available for only three of them). It is interesting to note, however, that the mean larval density used in the three studies that found *Plasmodium* to be virulent [15–17] was almost five times that of the two that did not [24,25] (mean larval density in studies that showed no effect = 113 larvae per litre, mean larval density in studies that showed an effect = 555 larvae per litre). This variation is likely to be important because even a fourfold increase in larval density can generate a significant decrease in adult body size [45], a prime determinant of mosquito survival [46]. Small mosquitoes produced from high-density larval conditions might have fewer resources to combat losses imposed by parasites. Experimental tests of the relationship between larval density and susceptibility to *Plasmodium* would be valuable.

Choice of control

Early research on *Plasmodium*–mosquito associations was mostly observational. Several papers published before 1950 did not use an uninfected control but asserted their results anecdotally [18,21,23]. Despite this, these studies are still cited as evidence for the neutrality of *Plasmodium* on their vector (e.g. Refs [13,27,28]). Among the studies with controls, there is still an important distinction in the type: (1) mosquitoes fed on uninfected blood or (2) mosquitoes without parasites regardless of infection status of earlier bloodmeals. Field-based studies must use the latter because it is not possible to ascertain the feeding history of wild-caught mosquitoes [27,30]. However, using this type of control group could be misleading if pathology associated with infection arises not from parasite development but from the toxicity and/or poor nutrient quality of infected blood. If mosquitoes fed on infected blood have lower survival even when oocysts and sporozoites do not develop, field-based studies might underestimate the effect of malaria on vector survival. Our sample has too few studies that have used the 'no sporozoite' design ($n = 2$) to draw any conclusions.

Genetic diversity

Field studies of *Plasmodium falciparum* and *Plasmodium vivax* have shown that each species harbours an enormous amount of genetic diversity for traits that mediate infection (e.g. merozoite surface

proteins) [47]. However, most laboratory studies of vector–parasite interactions use only one species of inbred mosquito and one genetically homogeneous *Plasmodium* strain. Studies of other invertebrates and their parasites have shown that susceptibility to infection depends on parasite genotype [48,49]. These results have recently been corroborated for malaria, when we showed that the survival of mosquitoes infected with *Plasmodium chabaudi* varied with parasite genotype [17]. One genotype was apparently benign, whereas another reduced mosquito survival (these effects are averaged in Table 2). Thus, a single study captures the contrasting conclusions of different studies. Results from field studies are not limited by genetic homogeneity because wild-caught mosquitoes are infected with many different parasite genotypes [50]. However, averaging the consequences of infection over many parasite genotypes might obscure important variation in pathogenicity.

Conclusions

We have identified several possible reasons why some studies have found that vectors are killed by malaria parasites whereas others have not. Studies reporting detrimental effects typically involve experiments of longer duration and unnatural vector–parasite combinations, and there might also be an effect of humidity. However, it is not easy to disentangle these various factors because they are confounded in studies to date. For example, the longest studies have been done on unnatural vector–parasite combinations (mean of 35.9 days for unnatural combinations vs 15.1 days for natural combinations, $F_{1,20} = 5.38$, $P = 0.03$), and the laboratory reporting the strongest effects [13,14] is also the one that maintained its mosquitoes in the driest atmosphere. Rather than overextend the statistics of meta-analysis, we suggest that experimental manipulations in a single laboratory are required to disentangle these factors and others, such as the role of genetic diversity.

In any case, are data on mosquito longevity in laboratory cages relevant to the natural transmission setting? Critics have suggested that the frequent use of unnatural vector–parasite combinations will generate artificially high virulence [30], and that laboratory experiments exclude possible indirect costs of infection such as increased risk of predation and feeding-associated mortality [42]. Our meta-analysis is certainly consistent with the first objection, although further experimental tests are required to separate the effects of co-evolutionary history and experimental design.

The importance of the second issue (failure to incorporate indirect effects) is unclear. Certainly, mosquitoes face a diverse array of environmental and biotic hazards in the field, most of which are eliminated within laboratory cages. Host defensive behaviour, for example, is a potentially significant source of mortality for mosquitoes [51], yet none of the studies that we have reviewed has allowed the possibility of post-infection anti-vector behaviour. Indirect mortality costs are probably higher for infected mosquitoes. It has been shown that, under natural field conditions, mosquitoes with sporozoites have greater feeding-associated mortality than those without sporozoites [42]. Laboratory results support this observation, confirming that infected mosquitoes are more persistent feeders [41] and have poorer flight ability [52,53], a trait that might reduce their ability to evade a defensive behaviour.

Short of unethical mark–recapture experiments of experimentally infected mosquitoes, it will be difficult to estimate the relative importance of the longevity effects detected in the laboratory and those that are a result of natural host feeding. The most plausible way forward is for experiments in the laboratory to incorporate these other possible sources of mortality. We know of only one study in which there was simultaneous estimation of the survival of a population when kept in cages and living free [27]. Interestingly, this study found survival to be higher in the free-living population, suggesting that the protective benefits of being in a cage might be outweighed by other factors. Only in the laboratory can mortality be properly examined over a range of conditions, such as variation in number of blood meals and environmental stress. Setting laboratory results into the context of the mortality experienced during a natural transmission cycle will require substantially more research, in both the laboratory and the field.

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Websites of interest

Medicines for Malaria Venture (MMV) website

The history of malaria can be found online at: **http://www.mmv.org**

This website outlines the new scientific disciplines and recent technologies, which can advance malaria drug research and development.

The site will be continually updated and will represent a source for the latest news on the disease.

***Anopheles* website**

Are you interested in the chromosomes of *Anopheles gambiae* and *Anopheles funestus*?
Or in methods for single-pair matings? Or how to prepare eggs for shipping, collecting pupae, separating them by sex and preparing larval food?
All this information is available at: **http://www2.ncid.cdc.gov/vector/vector.html**
You can also find this information at the Malaria Research and Reference Reagent Resource Center (MR4) website: **http://www.malaria.mr4.org** (select "*Anopheles* info")
If you have any methods or materials that others might benefit from, send them to: Mark Benedict at **mqb0@cdc.gov**

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