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LETTER

The evolution of virulence and host specialization in malaria parasites of primates

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Abstract

Parasite virulence, i.e. the damage done to the host, may be a by-product of the parasite's effort to maximize its fitness. Accordingly, several life-history trade-offs may explain interspecific differences in virulence, but such constraints remain little tested in an evolutionary context. In this phylogenetic study of primate malarias, I investigated the relationship between virulence and other parasite life-history traits. I used peak parasitaemia as a proxy for virulence, because it reflected parasite reproductive success and parasite-induced mortality. Peak parasitaemia was higher in specialist than in generalist species, even when confounding life-history traits were controlled. While there was a significant phylogenetic relationship between the number of competitors per host and host specialization, peak parasitaemia was unrelated to within-host competition. Therefore, the key evolutionary factor that favours virulence is host specialization, and the evolutionary success of virulent parasites, such as *Plasmodium falciparum*, may be better understood when the trade-off in virulence between different hosts is considered. Such phylogenetic results may help us design better protection programmes against malaria.

Keywords

Host specialization, malaria, *Plasmodium*, Primates, virulence.

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INTRODUCTION

Virulence incurs both benefits and costs. Virulent pathogens can achieve successful reproduction and transmission, but on the other hand, they need to exploit their host. As the good health of the host is a major component of parasite fitness, natural selection should favour parasites that maximize their fitness by optimizing growth, reproductive and transmission rates and virulence (Anderson & May 1982; Bull 1994; Frank 1996; Levin 1996; Combes 1997; Ebert 1998). Many parasites cause substantial host morbidity and mortality, and the explanation of high virulence occurring in nature is a central challenge in evolutionary biology. Several hypotheses have been proposed to explain why pathogens often cause harm. Some emphasize the role of trade-offs between virulence and other life-history traits such as infectivity, transmissibility and reproduction, whereas others emphasize the role of the host or competition with other parasites as key factors in controlling virulence (Anderson & May 1982; Bull 1994; Frank 1996; Levin 1996; Combes 1997; Ebert 1998).

It is often overlooked that some parasites infect a broad range of organisms while others are extremely specific, thus a trade-off between virulence and host specialization may exist (Woolhouse *et al.* 2001; Gandon 2004). When adapting to their hosts, parasites have to cope with host immune systems that produce an extremely diverse array of molecules (Strand & Pech 1995; Riffkin *et al.* 1996; Møller *et al.* 2005). The development of evasion mechanisms for each additional host species must be costly (Combes 1997). Therefore, parasites infecting several hosts may achieve lower reproductive success per host than parasites that infect fewer or a single host. This trade-off has been modelled in a mathematical simulation that showed that if a generalist parasite does not increase its virulence in one host without decreasing it in another, the parasite may remain a generalist with intermediate virulence or it may specialize on one host with escalating virulence (Regoes *et al.* 2000). However, there is no

comparative evidence that more virulent parasites tend to have narrower host ranges than less virulent parasites. Numerous theoretical and experimental studies have addressed the role of within-host competition as well as constraints on transmission and host specialization, but whether such constraints act on a phylogenetic scale remains to be determined.

Malaria in vertebrates is caused by a mosquito-borne, protozoan parasite of the genus *Plasmodium* which uses the host's erythrocytes for asexual reproduction (Coatney *et al.* 1971). There is an extreme variation among *Plasmodium* species in terms of the clinical symptoms they evoke: some of them often kill their hosts (e.g. *P. falciparum*), while others may replicate without affecting the condition of the host (e.g. *P. inui*) (Coatney *et al.* 1971; Fooden 1994; Markell *et al.* 1999). In fact, malaria caused by *P. falciparum* is one of the worst scourges of mankind, causing approximately 2-million deaths annually worldwide, although other *Plasmodium* species are less virulent. The pathology of malaria is almost exclusively due to the asexual erythrocytic stage parasite (Coatney *et al.* 1971; Paul *et al.* 2003). The higher the proportion of red blood cells the parasites contaminate, the more resources they distract from the host. Consequently, the peak concentration of parasites (i.e. their reproductive success) is a signature of the harm they cause. Parasitaemias exceeding peak counts of 100 000 infected cells mm^{-3} of blood (*c.* 2–5% of erythrocytes) are often fatal, while malarias with low parasite levels remain subclinical (Coatney *et al.* 1971). In a rodent malaria model, clones that produce more asexual parasites are more virulent in terms of anaemia, weight loss and mortality they cause than clones with lower parasitaemias (Mackinnon & Read 2004). Although, virulence, by definition, is the parasite-induced host mortality rate (Bull 1994), interspecific differences in peak parasite levels can be assumed to reflect interspecific differences in parasite virulence.

Using primate malarias as a test case, I investigated the phylogenetic relationships between parasite life-history traits to determine their role in the evolution of virulence. Based on a literature survey, I systematically collected data on median peak parasite levels of different *Plasmodium* species from infection experiments. These infection studies involving more than 1000 animals/patients rely on standardized experimental protocols, and hence provide a unique experimental data set for comparative studies. If parasites cannot maintain high virulence when they simultaneously adapt to more than one host, there should be trade-off in virulence of malaria parasites between different hosts. Accordingly, natural selection should drive a negative interspecific relationship between estimates of host specialization and peak parasite levels, if the latter reflects virulence.

MATERIALS AND METHODS

Data set

Data on median peak parasite levels and various life-history traits of different *Plasmodium* species were derived from the literature (see Table S1).

I obtained information on median peak parasite levels from published experiments, in which malaria was induced in 12 model hosts (natural and non-natural) altogether. These infection experiments were performed blindly to the main hypothesis of the current study, thus potential methodological differences between laboratories should be subject to random noise in the present context. However, I applied strict selection criteria to reduce any potential noise in the data. I considered experiments that used untreated, intact (non-splenectomized, non-medicated) animals, but only if parasites were inoculated on a known infection day and then monitored for several weeks. Using multiple and quantitative information on peak parasite levels per species, I first performed analyses at the level of individual experiments. This approach allowed me to test for consistent variation within *Plasmodium* species and to control for the fact that different experiments used different host models. The control for host effects is important, because different hosts may differ in terms of malaria resistance or life history, thus peak parasitaemias (and virulence) may be partially dependent on the hosts' attributes. Additionally, natural and non-natural hosts may react differently to experimental infections. I analysed data at the level of experiments using a GLM approach. I built a linear model with median peak parasite levels as the dependent variable, and tested for the effect of parasite species (main effect), while involving host species and host type (natural vs. non-natural) as random factors. In a second model, I considered peak parasitaemias from natural hosts only and controlled for host species. From this second model, I calculated species-specific estimates of peak parasitaemias that are independent of host effects. Finally, I used these estimates in a set of phylogenetic analyses to test the determinants of peak parasitaemias at the interspecific level.

Host specialization was defined as the number of natural host species reported in the literature. Although such measures of specialization have been criticized (Poulin & Mouillot 2005), the intensive study of malaria in non-natural primate hosts allowed tests of biological relevance based on unique experimental data providing, at least, qualitative information on infection success. Several attempts, with variable success, have been made to grow different *Plasmodium* species in a variety of hosts (e.g. Coatney *et al.* 1971; Collins & Contacos 1979; Collins *et al.* 1987, 1990; Stewart 2003). The rate at which a parasite can successfully infect a wide array of experimental hosts is presumably

related to its host specialization. Thus, for each *Plasmodium* species, I compared the number of successfully infected non-natural host species (irrespective to parasite levels) to the number of all non-natural species tested. This ratio was positively and significantly related to the number of natural host species ($r = 0.48$, $P = 0.02$, $n = 23$). Additionally, the number of host species was strongly associated with the number of host genera ($r = 0.86$, $P < 0.001$, $n = 26$) and with the maximum phylogenetic distance between natural hosts as estimated from the phylogeny of primates ($r = 0.88$, $P < 0.001$, $n = 26$). Therefore, I assumed that the number of natural hosts of a malaria parasite reliably reflects the parasite's potential to infect new hosts, i.e. its host specialization. The number of natural host species was not significantly related to an estimate of research effort ($r = 0.26$, $P = 0.25$, $n = 26$), to parasite distribution ($r = 0.36$, $P = 0.07$, $n = 26$) and to parasite prevalence ($r = -0.06$, $P = 0.85$, $n = 12$), but it was significantly associated with host taxonomy at the higher level (differences between Platyrrhini–Cercopithecoidea–Hominoidea parasites: $F_{2,22} = 10.18$, $P < 0.001$). Based on these correlations, I cannot fully exclude the confounding effects of these variables (see also Krasnov *et al.* 2004). However, when their effects were controlled statistically the main findings of this paper were very similar (see Table S2).

I also used experimental data to assess mosquito susceptibility. In a set of transmission studies, the susceptibility of different species of *Anopheles* mosquitoes, the natural vectors of *Plasmodium* species, was expressed relative to a standard species, such as *Anopheles freeborni* or *A. balabacensis balabacensis*, in the form of Gut Infection Index (GII%). GII% is the average number of oocysts per 100 guts, compared with the standard mosquito that fed simultaneously with the species being compared. Within *Plasmodium* species and across different vector species, GII% was significantly repeatable ($F_{17,68} = 2.58$, $P = 0.01$, when sample size and variation between *Anopheles* spp. was held constant). GII% was positively and significantly related to the percentage of mosquitoes infected ($F_{1,74} = 29.55$, $P < 0.001$). Hence, GII% is a parasite-specific estimate of mosquito susceptibility and I assumed that it reflects the parasite's ability to spread gametocytes into different mosquitoes. Accordingly, for each *Plasmodium* species, I calculated least square GII% while controlling for vector species.

In each host of each parasite species, I determined the number of other *Plasmodium* species that naturally infect the same host and thus compete for the same resources (erythrocytes). Assuming that the presence of another species necessarily involves certain degree of competition, I used the average number of competitors as an estimate of within-host competition. Interspecific competition has been described in avian malaria parasites, which is a result of interference during parasite transmission, and may also occur between human malaria parasites (Paul *et al.* 2002). The number of competitors was highly consistent within *Plasmodium* species and across hosts ($F_{25,65} = 6.78$, $P = 0.01$).

I characterized within-host reproductive dynamics for each *Plasmodium* species. I used information on periodicity, as the time needed to complete one asexual cycle, which varies between 24 and 72 h (quotidian, tertian and quartan periodicity). To estimate within-cycle fecundity, I used the average number of merozoites (new, asexual parasites produced in one erythrocyte during one cycle, which were assumed to have similar ability to invade new cells). I also included data on the length of the pre-patent period, the number of days elapsing between infection and the detection of the parasite in the blood as measured in natural hosts.

From the literature, I also extracted information on mortality rate, capacity to relapse and prevalence. Mortality rate was defined as the number of infections with fatal outcome compared to the total number of infections. Capacity to relapse is the potential of a parasite to produce secondary infections after longer impatent periods, and was defined qualitatively. Symptomatic relapse is caused by reactivation of the dormant form of the parasite. Finally, prevalence was defined as the number of infected individuals found in relation to the number of all individuals examined.

The full data set is reported on the web site of the Journal as a supplementary table (see Table S1). All variables were transformed according to their distribution.

Phylogenetic methods

I calculated interspecific correlations between the phenotypic traits of parasites. However, sister taxa may be more similar with respect to the variables under investigation than randomly chosen species. Therefore, I investigated the phylogenetically corrected relationship between traits by using Generalized Least Squares models implemented in the program Continuous (Pagel 1999). For this evolutionary modelling, relying on phylogenetic studies that used molecular techniques (Escalante *et al.* 1998; Perkins & Schall 2002; Leclerc *et al.* 2004; Escalante *et al.* 2005), I constructed two phylogenetic trees of primate malaria parasites (Fig. 1a, c). The first tree included those 13 species for which information on peak parasitaemias were available, while the second tree contained those 22 species that were used in the analyses of host specialization (note that not all the 26 species were used in these analyses). Although different sources report slightly different phylogenetic relationships for certain taxa (e.g. the position of *P. ovale* and the relationships within the *vivax*-group), and the phylogenetic relationships of some species considered are

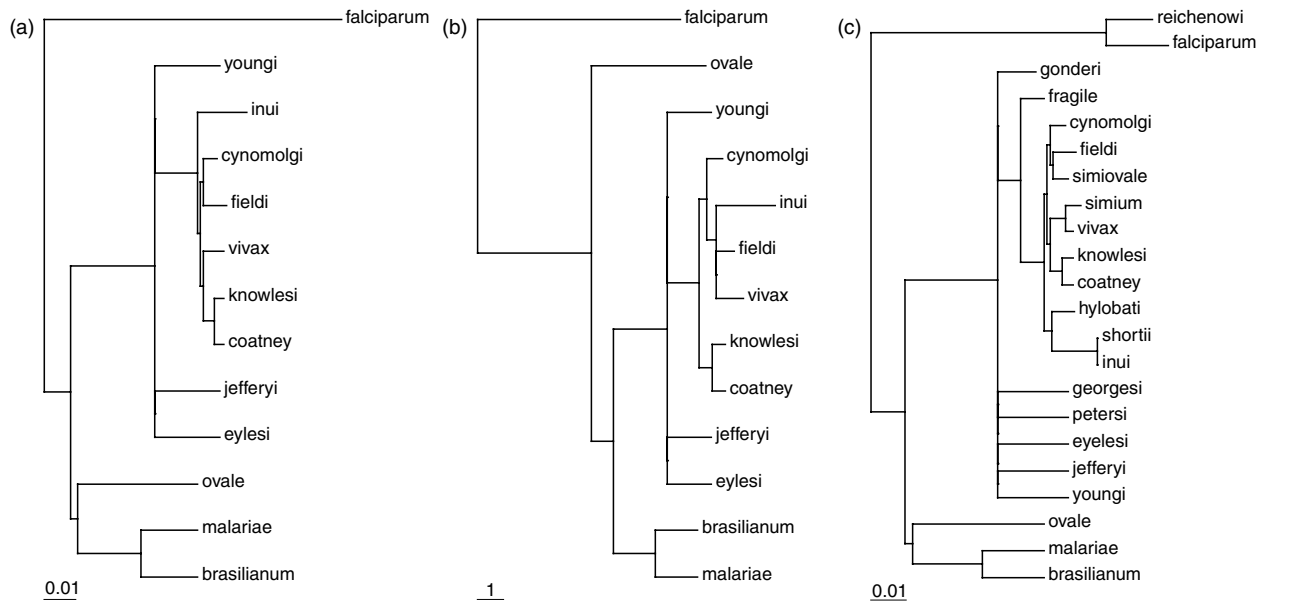

Figure 1 Phylogenetic hypotheses of *Plasmodium* species of primates used to estimate the phylogenetically corrected relationships between traits and to assess the mode of trait evolution by using Least Square models. (a) Phylogenetic tree used in the interspecific analyses of peak parasitaemias (13 species), based on Escalante *et al.* (1998), phylogenetic information for *Plasmodium brasilianum*, *Plasmodium fragile* and *Plasmodium coatney* were added from Leclerc *et al.* (2004) and Escalante *et al.* (2005); (b) an alternative phylogenetic hypothesis that was based on Perkins & Schall (2002) and provides very similar comparative results; (c) the phylogenetic hypothesis of species ($n = 22$) that were used to investigate the relationship between host specialization and within-host competition, based on Escalante *et al.* (1998, 2005) and Leclerc *et al.* (2004). The scales for branch lengths in the bottom left corners correspond to the estimated phylogenetic distances as provided by the reference sources. (a) and (c): Faltier and Fouy distance; (b): branch lengths are proportional to the number of nucleotide changes.

not known, any uncertainty in the phylogeny should be independent of the topic at hand. However, to deal with unresolved phylogenetic relationships, I allowed polytomies on the phylogenetic trees and tested the effect of an alternative phylogeny (Fig. 1b), which provided very similar results. When modelling the mode of trait evolution, I generally found no role for phylogenetic inertia ($\lambda \approx 0$) and evidence for a punctuational mode of trait evolution ($\kappa \approx 0$) (Pagel 1999). I present phylogenetic correlations (r_{phyl}) and the significance of the associated likelihood ratio statistics that were calculated after adjusting for the mode of evolution of traits at hand. However, due to the absence of phylogenetic inertia, conclusions from analyses with and without phylogenetic adjustment are identical.

RESULTS

Intraspecific variation in peak parasitaemias

There was a consistent variation in median peak parasite levels between *Plasmodium* species, when host effects were controlled ($F_{16,49} = 7.24$, $P < 0.001$). Therefore, variation in median peak parasite levels, which may be caused by differences between laboratories or parasite clones for instance, seems negligible compared with the variation between species. Median peak parasitaemias are parasite-specific attributes, and comparisons of species based on species means make biological sense.

The model revealed significant effect for host species ($F_{11,49} = 29.4$, $P < 0.001$) suggesting that peak levels vary systematically among host species and are partially dependent on the host. Moreover, peak parasite counts were affected by host naturality, because peak parasitaemias were higher in natural hosts than in non-natural hosts ($F_{1,49} = 68.4$, $P < 0.001$). Limited information on the mode of infection was available, but it did not associate with peak levels (sporozoite- vs. blood-induced peak: $F_{1,31} = 0.15$, $P = 0.70$).

When I excluded experiments on four non-natural hosts and controlled for the effect of host species, the effect of parasite species was also very strong ($F_{12,26} = 5.68$, $P < 0.001$). This model explained most of the variance in peak parasitaemias ($r^2 = 0.91$) and corresponded better to the natural situation than models including non-natural hosts. Therefore, from this less-parameterized model, I determined the least square means of median peak parasitaemias (hereafter LS peak parasitaemias) from eight natural hosts by controlling for the effect of host species. I used LS peak parasitaemias in the interspecific analyses as *Plasmodium*-specific estimates that are independent of

between-host variation and host naturality. Note that least square means can be estimated with a certain error.

Interspecific determinants of peak parasitaemias

Associations with estimates of within-host reproductive dynamics revealed that LS peak parasitaemias reflect how parasites grow in their hosts (periodicity: $r_{phyl} = -0.52$, $P = 0.04$, $n = 13$; within-cycle fecundity: $r_{phyl} = 0.61$, $P = 0.01$, $n = 13$; pre-patent period: $r_{phyl} = -0.69$, $P < 0.01$, $n = 11$). Furthermore, across the few species for which data are available, LS peak parasitaemia was positively related to malaria-induced host mortality rate ($r_{phyl} = 0.78$, $P < 0.01$, $n = 8$). Hence, LS peak parasitaemia is a marker of virulence, but it *also* mirrors an entire suite of life-history variables describing reproductive mechanisms (see also Introduction).

I found a negative phylogenetic relationship between LS peak parasitaemias and the degree of host generalization ($r_{phyl} = -0.71$, $n = 13$, $P < 0.01$, Fig. 2), i.e. specialists parasites were more virulent. I assessed the potentially confounding effects of within-host competition, mosquito susceptibility and the capacity to relapse. Although mosquito susceptibility was significantly related to LS peak parasitaemias when host specialization was held constant

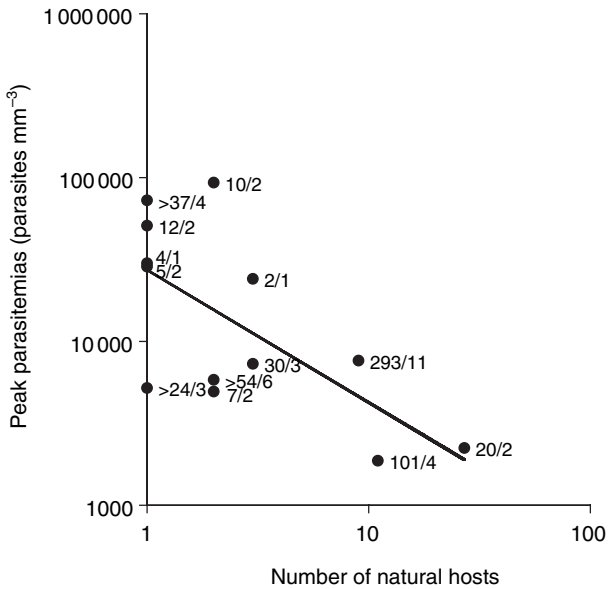

Figure 2 The interspecific association between host specialization and peak parasitaemias after controlling for the variation between hosts by least square means (see main text). Data points ($n = 13$) are summary statistics for different *Plasmodium* species usually involving data from more than one infection experiment. Numbers indicate sample size in terms of the number of animals infected/number of experiment when involving natural hosts only. Raw species data are presented.

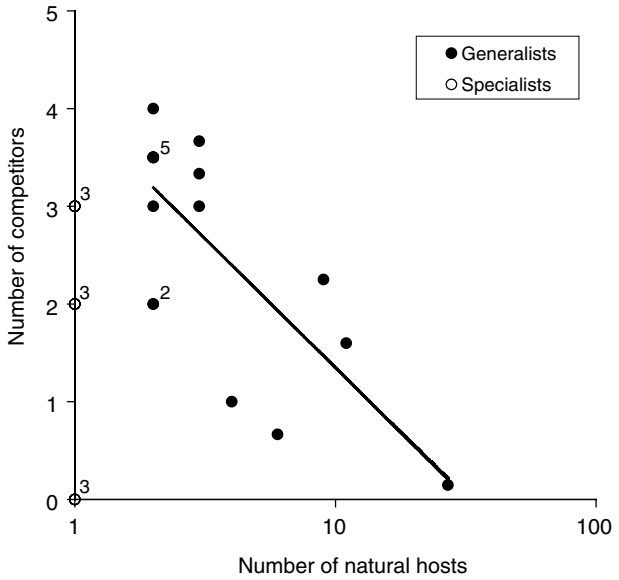

Figure 3 Host specialization and within-host competition in primate malarias (*Plasmodium* spp). The regression line is fitted for generalist species only (species infecting single hosts are excluded). Numbers indicate sample size in terms of the number of species for the overlapping points. Raw species data are presented.

(partial correlations, number of competitors: $r_{phyl} = -0.15$, $n = 13$, $P = 0.62$; mosquito susceptibility: $r_{phyl} = -0.70$, $n = 12$, $P = 0.02$; capacity to relapse: $r_{phyl} = -0.59$, $n = 10$, $P = 0.10$), the relationship between LS peak parasitaemias and host specialization was independent of the potentially confounding factors ($-0.86 < r_{phyl} < -0.66$, $P < 0.05$).

There appeared to be a phylogenetic relationship between host specialization and within-host competition. After excluding specialist species infecting a single host, the number of competitors per host was significantly and negatively related to the number of host species ($r_{phyl} = -0.84$, $n = 15$, $P < 0.001$; Fig. 3).

DISCUSSION

In this phylogenetic study of primate malarias, I showed a negative phylogenetic relationship between LS peak parasitaemias and host specialization. This pattern indicates that *Plasmodium* parasites apparently cannot reproduce with high success in hosts over a broad evolutionary spectrum, and thus host adaptation may involve some costs. Therefore, there is a trade-off between reproductive success and the number of hosts. As LS peak parasitaemias predicted host mortality rate (see also Mackinnon & Read 2004), and the number of hosts may reflect the parasite's ability to infect new hosts, the negative association between LS peak parasitaemias and the number of host species also implies

a trade-off between host specialization and parasite virulence. According to my knowledge, this comparative study using real experimental data is the first empirical report that shows the existence of such evolutionary constraint.

Intensive study of *P. falciparum* revealed some aspects of the sophisticated molecular mechanisms that parasites apply when adapting to a host, which may shape the observed evolutionary patterns. For example, several multigene families are involved in immune evasion, which govern clonal antigenic variation of surface molecules that are important targets of the humoral immune response of the host (Kyes *et al.* 2001). As such adaptation relies on the interplay with the host's specific recognition ability, it requires a certain degree of specialization. Indeed, a mathematical model predicts that intragenomic interactions between parasite loci encoding antigenic peptides are pivotal in determining the outcome of evolution of generalist and specialist parasite genotypes (Hamilton *et al.* 2005). Parasites that are very successful in this complex molecular adaptation will be able to reproduce at a high rate and thus become virulent in a certain host. However, by their extreme specialization, they may lose their ability to fit the variation of surface molecules to the immune system of other hosts, and thus become unable to grow. Interestingly, some of the molecules showing antigenic variation are also major virulence factors that mediate adhesion to a variety of host cell types (Kyes *et al.* 2001). Again, such mechanisms also require an active signalling process between hosts and parasites favouring host specialization, which also implies a trade-off between host specialization and virulence.

I assessed the robustness of the relationship between host specialization and virulence by examining whether this association was mediated by a third factor. Theoretically, the effect of within-host competition is important to separate out, because it can lead to a covariation between virulence and specialization without a causal relationship. For example, restricted host range, by influencing the probability of encounters with other parasite species, may shape within-host competition (Combes 1997), which in turn can have an impact on parasite virulence (Frank 1996; Mosquera & Adler 1998). The number of host species was related to number of competitors per host, but only after excluding specialist species infecting a single host. Therefore, malaria parasites that already have the flexibility to grow in at least two different hosts experience reduced interspecific competition, if they increase host range on an evolutionary time scale. However, peak parasitaemias were unrelated to the number of competitors per host. This implies that although intraspecific competition between clones is likely to be intense and shape virulence (De Roode *et al.* 2003, 2005), interspecific competition does not necessarily have similar evolutionary consequences. Competition between *Plasmodium* species may be weak, if these parasites exploit different niches within host and they elicit specific immune responses. Altogether, if within-host competition is an important selection force in primate malarias, on the monitored evolutionary time scale, it affects host specialization rather than parasite virulence.

I also determined the effects of mosquito susceptibility and the capacity to relapse, and the former trait was significantly and negatively related to LS peak parasitaemias. Virulent malaria parasites maintaining high titres can reach high transmission rates and can enhance mosquito fecundity (Schall 2000; Ferguson *et al.* 2003). Perhaps this increased transmission success favours virulent parasites to use few mosquito species only. Therefore, the trade-off between specialization and virulence may also apply to vector specialization. Most importantly, the phylogenetic relationship between virulence and host specialization was unaffected by mosquito susceptibility and the capacity to relapse. Therefore, the key selection force shaping the reproduction of *Plasmodium* species in their hosts that ultimately reflects their virulence seems to be the extent to which they adapt to different hosts.

As comparative studies are based on correlations and are constrained by data availability, these limitations warrant some attention. First, the definition and the use of the marker variables were conditional to certain assumptions. While, some of these could be tested, others were merely supported by scientific speculation. Second, although the raw data were derived from more than 1000 experiments, species-specific LS parasitaemias could be estimated with a certain error, and the number of parasite species limited the power of the interspecific tests. However, these problems are more likely to raise random errors than systematic bias. Third, although the detected pattern supports the hypothesis that host specialization constrains virulence (or vice versa) on an evolutionary scale, this does not necessarily mean that every virulent parasite is specialist, because additional factors may also favour virulence [e.g. the generalist *Plasmodium relictum* has led to the extinction of many bird species (Van Riper *et al.* 1986)]. I assessed the effect of several potentially confounding variables (see Results and Table S2), but in a comparative study, other factors accounting for some additional variance may remain unidentified, e.g. due to the lack of data. For example, it would be interesting to test how the duration of the evolutionary contact between parasites and host shapes virulence. Fourth, the phylogeny and taxonomy of the genus *Plasmodium* are subject to some debate (e.g. see Fig. 1). Although I showed minor roles for phylogenetic inertia, the results may be sensitive to taxonomic considerations. For example, *Plamodium brasilianum* and *Plasmodium malariae* are sometimes regarded as a single species (Cochrane *et al.* 1985; Escalante *et al.* 1995; Fandeur *et al.* 2000). However, when I reanalysed the data by adopting

this species concept, the results did not change (LS parasitaemias–host specialization: $r = -0.587$, $P = 0.045$, $n = 12$).

Despite these potential shortcomings, my phylogenetic findings have important implications for evolutionary parasitology because they are the first comparative evidence that the evolution of virulence and host specialization is mediated by the same selection factors. Here I used the combination of experimental and comparative methods, an approach that is rarely used (e.g. Møller 2000; Arnqvist & Rowe 2002; Rowe & Arnqvist 2002), but has the potential to assess directly the evolutionary consequences of biological effects. In addition, the evolutionary ecology of *Plasmodium* parasites highlights potentially fruitful avenues of research for developing malaria control strategies (Paul *et al.* 2003; Mackinnon & Read 2004). My phylogenetic modelling revealed that the coupled evolution of peak parasitaemias and host specialization is independent of common ancestry, and a role for a punctuational mode of evolution (see Materials and methods). Hence, these traits are not constrained by phylogenetic relationships, and evolutionary steps can occur relatively fast. Consistently, laboratory tests and theoretical models suggest that the rate of adaptation of malaria parasite populations can be remarkably high with the half-time of virulence being less than 40 years (Mackinnon & Read 2004). Such quick adaptation may guarantee the success of protection programmes that are based on evolutionary concepts (Paul *et al.* 2003; Mackinnon & Read 2004), in which the current knowledge about the trade-off between parasite virulence and host specialization could be implemented.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available online from http://www.Blackwell-Synergy.com:

Table S1 Phenotypic and life-history traits of malaria parasites of primates.

Table S2 Associations traits of interest when holding potentially confounding effects constant.

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