Stress, drugs and the evolution of reproductive restraint in malaria parasites

Citation for published version:
Reece, SE, Ali, E, Schneider, P & Babiker, HA 2010, 'Stress, drugs and the evolution of reproductive restraint in malaria parasites' Proceedings of the Royal Society B-Biological Sciences, vol 277, no. 1697, pp. 3123-3129. DOI: 10.1098/rspb.2010.0564

Digital Object Identifier (DOI):
10.1098/rspb.2010.0564

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Proceedings of the Royal Society B-Biological Sciences

Publisher Rights Statement:
RoMEO green

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Stress, drugs and the evolution of reproductive restraint in malaria parasites

Sarah E. Reece¹,²,* Eltayeb Ali³,⁴ Petra Schneider¹,²
and Hamza A. Babiker²,⁵

¹Centre for Immunity, Infection and Evolution, and ²Institutes of Evolution, Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3JT, UK
³Sudan Atomic Energy Commission, PO Box 3001, Khartoum, Sudan
⁴Department of Biochemistry, Faculty of Medicine, University of Khartoum, Khartoum, Sudan
⁵Biochemistry Department, Faculty of Medicine, Sultan Qaboos University, Alkhod, PO Box 35, Muscat, Oman

Life-history theory predicts that sexually reproducing organisms have evolved to resolve resource-allocation trade-offs between growth/survival versus reproduction, and current versus future reproduction. Malaria parasites replicate asexually in their vertebrate hosts, but must reproduce sexually to infect vectors and be transmitted to new hosts. As different specialized stages are required for these functions, the division of resources between these life-history components is a fundamental evolutionary problem. Here, we test how drug-sensitive and drug-resistant isolates of the human malaria parasite Plasmodium falciparum resolve the trade-off between in-host replication and between-host transmission when exposed to treatment with anti-malarial drugs. Previous studies have shown that parasites increase their investment in sexual stages when exposed to stressful conditions, such as drugs. However, we demonstrate that sensitive parasites facultatively decrease their investment in sexual stages when exposed to drugs. In contrast to previous studies, we tested parasites from a region where treatment with antimarial drugs is common and transmission is seasonal. We hypothesize that when exposed to drugs, parasites invest in their survival and future transmission by diverting resources from reproduction to replication. Furthermore, as drug-resistant parasites did not adjust their investment when exposed to drugs, we suggest that parasites respond to changes in their proliferation (state) rather the presence of drugs.

Keywords: Plasmodium falciparum; life-history trade-offs; gametocyte conversion; anti-malarial drug resistance; reproductive effort; resource allocation

1. INTRODUCTION

In its broadest sense, phenotypic plasticity is the ability of a single genotype to produce different phenotypes in different environments, through mechanisms such as differential gene expression or epigenetic effects (Schlichting & Pigliucci 1998). Phenotypic adjustment can occur in response to changes in an individual’s extrinsic environment (e.g. appearance of predators) or intrinsic state (e.g. parasite burden) and is considered to be adaptive when it results in increased fitness, through enhanced survival or reproductive success (Schlichting & Pigliucci 1998). The extent to which individuals can adjust their behaviours or express different combinations of traits is constrained by resource-allocation trade-offs in which organisms have to split their finite resources between the multiple processes (e.g. growth/survival, reproduction) required to transmit gene copies to subsequent generations (Roff 1992; Stearns 1992). Life-history theory provides a solid foundation for understanding plasticity in resource-allocation trade-offs and can be used to explain and predict the evolutionary consequences of environmental variation (Roff 1992; Stearns 1992; Fischer et al. 2009; McNamara et al. 2009).

While adaptive phenotypic plasticity has been thoroughly documented for metazoan taxa (such as insects, birds and mammals), this concept has been largely overlooked for unicellular taxa such as malaria (Plasmodium) parasites (Reece et al. 2009). Malaria parasites face many analogous selective pressures and resource trade-offs to metazoans, and data suggest they also have considerable plasticity in their resource-allocation decisions (Paul et al. 2000; Reece et al. 2008, 2009). The value of an evolutionary approach for understanding how variation in the in-host environment shapes within-infection dynamics and contributes to virulence and transmission traits is increasingly being recognized. Malaria parasites replicate asexually in the circulation of their vertebrate hosts, but must produce male and female sexual stages (termed gametocytes) to transmit to vectors and subsequently infect new hosts. As in-host replication and between-host transmission are each achieved by different specialized forms, the division of resources between these life-history components is a fundamental evolutionary problem (Koella & Antia 1995). This is analogous to the problem faced by sexually reproducing organisms, in which the trade-off between investing resources into reproduction (reproductive effort) relative to growth and maintenance is a major determinant of fitness (Roff 1992; Stearns 1992).
Experiments have revealed that malaria parasites of rodents and humans increase their investment in gametocytes in response to host anaemia, changes in the age of available red blood cells (RBCs) and exposure to antimalarial drugs (Trager & Gill 1992; Buckling et al. 1997, 1999a,b; Buckling & Read 1999; Trager et al. 1999; Reece et al. 2005). These observations have been interpreted as environmental changes that are stressful for parasites, to which they respond by diverting resources to gametocytes to increase between-host transmission (Taylor & Read 1997; Read & Taylor 2001). This ‘terminal investment’ is predicted by evolutionary theory when changes in intrinsic state, the environment or availability of resources reduce the probability of survival (Williams 1966). However, changes in the age of available RBCs can result in parasites having more useable resources (Paul & Brey 2003; Paul et al. 2003; Reece et al. 2005), and parasites adopt the opposite to terminal investment and are predicted to decrease investment in gametocytes in response to competitive suppression in mixed infections (Mideo & Day 2008; Pollitt et al. submitted). Therefore, parasites do not necessarily respond to stress by increasing investment in gametocytes, and in situations where ‘safety in numbers’ can facilitate in-host survival, ‘reproductive restraint’ is a better investment decision (Mideo & Day 2008; Reece et al. 2009; Pollitt et al. submitted). Here, we focus on testing whether parasites also adopt reproductive restraint when treated with antimalarial drugs that suppress their proliferation.

The suggestion that malaria parasites alter investment into gametocytes when exposed to drugs has received mixed support. However, research has focused solely on whether investment is increased or not adjusted (Puta & Manyando 1997; Buckling et al. 1999; Trager et al. 1999). These observations have been interpreted as environmental changes that are stressful for parasites, to which they respond by diverting resources to gametocytes to increase between-host transmission (Taylor & Read 1997; Read & Taylor 2001). This ‘terminal investment’ is predicted by evolutionary theory when changes in intrinsic state, the environment or availability of resources reduce the probability of survival (Williams 1966). However, changes in the age of available RBCs can result in parasites having more useable resources (Paul & Brey 2003; Paul et al. 2003; Reece et al. 2005), and parasites adopt the opposite to terminal investment and are predicted to decrease investment in gametocytes in response to competitive suppression in mixed infections (Mideo & Day 2008; Pollitt et al. submitted). Therefore, parasites do not necessarily respond to stress by increasing investment in gametocytes, and in situations where ‘safety in numbers’ can facilitate in-host survival, ‘reproductive restraint’ is a better investment decision (Mideo & Day 2008; Reece et al. 2009; Pollitt et al. submitted). Here, we focus on testing whether parasites also adopt reproductive restraint when treated with antimalarial drugs that suppress their proliferation.

The suggestion that malaria parasites alter investment into gametocytes when exposed to drugs has received mixed support. However, research has focused solely on whether investment is increased or not adjusted (Puta & Manyando 1997; Buckling et al. 1999b; Drakeley et al. 2006; Babiker et al. 2008; Peatey et al. 2009), and the alternative possibility of decreased investment (reproductive restraint) has been overlooked. Furthermore, previous studies have focused on short exposures at high doses that are insufficient to clear infections but still cause a rapid decline in parasite numbers. If rapid decline in numbers is a reliable cue of a catastrophic situation, the potential for future transmission is low, and so parasites are expected to adopt terminal investment. Here, we test whether multiple lines of the human malaria parasite Plasmodium falciparum adopt reproductive restraint in response to exposure to two different antimalarial drugs that target asexual replication. We focus on providing continuous sublethal stress by treating parasites with low doses of drugs across multiple replication cycles. In endemic areas, malaria parasites face low levels of anti-malarial drugs for a variety of reasons. For example, recommended regimes can fail to eliminate parasites because treatment success is influenced by parasite density, and patients can be reininfected before previous treatments are cleared (Adjuik et al. 2004; Mutabingwa et al. 2005; Barnes et al. 2007). The latter is especially likely under intermittent preventive treatment, which involves giving therapeutic doses to vulnerable groups (infants and pregnant women) at regular intervals, regardless of infection status. In contrast to previous studies, we also tested parasite lines isolated from natural infections in a population where exposure to anti-malarial drugs is frequent. We used drug-sensitive and drug-resistant lines to test whether parasites alter their investment as a general response to the presence of drugs or only when vulnerable to drug treatment.

2. MATERIAL AND METHODS
(a) Parasites and cultures
The initial isolates were obtained with informed consent from patients in Asar village in Eastern Sudan. In this region, malaria transmission is markedly seasonal, following the rainy season (September to November). Plasmodium falciparum is the predominant parasite species, and treatment with chloroquine and/or pyrimethamine is frequent in the transmission season (Babiker et al. 1991). We established each isolate in in vitro culture according to Jensen & Trager (1977), using narrow-necked culture flasks. Briefly, each culture consisted of 5 ml complete culture medium, containing RPMI supplemented with 25 mM NaHCO3 (Sigma, UK), 25 mM 4-(2-hydroxyethyl)-piperazinethane-sulfonic acid (Sigma, UK), 10 per cent naive human serum, adjusted to pH 7.4, gassed with a mixture of 3 per cent O2, 5 per cent CO2 and 92 per cent N2, and sealed throughout incubation. We obtained distinct experimental lines by limiting dilution from isolates exhibiting diversity for genetically controlled traits including sensitivity to chloroquine and pyrimethamine (Babiker et al. 1991; Bayoumi et al. 1993). We obtained three lines sensitive to both chloroquine and pyrimethamine (identity codes: 107/89, 109/89, 104/89) and three lines resistant to both drugs (124/8, 121/89, 123/89).

(b) Experimental procedures
We established three replicate cultures for each of our three drug-sensitive and three drug-resistant lines, and allocated one replicate per line to each of the following treatments: no-drug control, chloroquine-treated and pyrimethamine-treated. Therefore, we followed a cross-factorial design in which parasites from each line experienced all experimental treatments. We set up each line/treatment combination in duplicate or triplicate, resulting in six to nine cultures for each of our six lines, totalling 39 cultures. We followed Carter et al. (1993) to establish gametocyte-producing cultures: as soon as 5 per cent of RBCs were parasitized, 1 ml (5%) was added to 4 ml of 6 per cent haematocrit-uninfected blood, in media appropriate to the treatment group for each culture (media without drugs, with 0.5 pmol chloroquine or with 0.5 nM pyrimethamine) and the haematocrits of all cultures were set to 6 per cent (no further RBCs were added during culturing). The doses of chloroquine and pyrimethamine we used are low: the minimum doses required to clear infections, in media appropriate to the treatment group for each culture (media without drugs, with 0.5 pmol chloroquine or with 0.5 nM pyrimethamine) and the haematocrits of all cultures were set to 6 per cent (no further RBCs were added during culturing). The doses of chloroquine and pyrimethamine we used are low: the minimum doses required to clear all sensitive parasites are 8 pmol and 10−5 M, respectively. We maintained cultures for 11 days and replaced culture medium daily with either drug-free or drug-containing media according to the treatment for each culture. We sampled cultures daily using Giemsa-stained thin blood smears and recorded the parasitaemia, the numbers of ring stages and stage 2 gametocytes per 10 000 RBCs. We used these values to calculate the gametocyte conversion rates for each culture from day 0 to day 8, following Carter & Miller (1979). The gametocyte conversion rate (reproductive effort) represents the proportion of a cohort of asexually produced ring-stage parasites that become sexual-stage gametocytes. After invading an RBC, parasites spend their first 24 h as ring stages, which can be readily distinguished in blood smears. Gametocytes cannot reliably be distinguished by morphology until they have reached stage 2 of
Table 1. Model estimates (± s.e.) and significance for the intercepts and slopes for transformed conversion rates examined in our second analysis, revealing that the conversion rates of sensitive (vulnerable) parasites exposed to drugs are significantly lower than for other treatment groups (safe from drugs), and that conversion rates of parasites safe from drugs were not significantly different from each other. Estimates (± s.e.) given are relative to the conversion rates of the resistant parasites in control cultures, and t- and p-values test the difference between the resistant parasites in control cultures and the other groups.

|                | t   | p    |
|----------------|-----|------|
| intercepts     |     |      |
| resistant parasites without drugs | -3.545 (± 0.25) | 0.0578 |
| Δ resistant parasites exposed to drugs | 0.166 (± 0.30) | 0.56   |
| Δ sensitive parasites without drugs | 0.242 (± 0.37) | 0.66   |
| Δ sensitive parasites exposed to drugs | 0.400 (± 0.44) | 0.91   |
| slopes         |     |      |
| resistant parasites without drugs | 0.278 (± 0.04) | 0.901  |
| Δ resistant parasites exposed to drugs | -0.007 (± 0.05) | 0.15   |
| Δ sensitive parasites without drugs | -0.007 (± 0.06) | 0.12   |
| Δ sensitive parasites exposed to drugs | -0.167 (± 0.07) | 2.41   |

their development, 48 h after invasion. Therefore, the conversion rate on day \( t \) is simply calculated as the number of stage 2 gametocytes observed in 10,000 RBCs on day \( t + 2 \) divided by the number of ring-stage asexual parasites observed in 10,000 RBCs on day \( t \). Unlike in vivo infections, in vitro culture is ideal for calculating conversion rates because all parasites of all developmental stages are accessible for sampling (gametocytes cannot sequester in tissues and asexuals are not exposed to attack from immune factors), so potential problems such as stage-specific mortality cannot confound estimates.

(c) Statistical analysis
We used R v. 2.5.0 (The R Foundation for Statistical Computing, Vienna, Austria) for all analyses. We log-transformed conversion rates to conform to the assumptions of parametric tests and used linear mixed-effects models with maximum likelihood to investigate the effects of drug treatment and drug sensitivity on the patterns of conversion rates observed during cultures, according to the three sets of analysis described below. Mixed-effects models allowed each culture to be nested within the identity of the line contributing the parasites while fitting line identity as a random effect, which can account for problems of non-independence associated with repeated measures in longitudinal analyses. We nested culture within the identity of each line for all analyses and evaluated the significance of fixed effects by comparing models using log-likelihood ratio tests following stepwise deletion of the least significant term. Specifically, we compared the change in model deviance, following term deletion, to \( \chi^2 \) distributions with degrees of freedom corresponding to the difference in the number of terms in the models. We simplified maximal models until only significant terms remained in the model (Pinheiro & Bates 2000). Minimal models from our different analyses were compared with log-likelihood ratio tests and Akaike information criteria (AIC). We then re-ran our final minimal model using restricted maximum likelihood to estimate the effect sizes and plot model predictions.

We ran three sets of models to find the most parsimonious description for the influences of drug treatment and susceptibility to drugs on the patterns of gametocyte conversion rates observed during cultures. By comparing models in which we combined different factor levels and different treatment groups, we tested whether gametocyte conversion rates differed between cultures according to the type of drug used and the susceptibility of parasites. In addition to testing for the effects of drug treatment or drug sensitivity, we also fitted RBC density as a covariate in all maximal models as host anaemia has been shown to influence conversion rate during in vivo infections (Reece et al. 2005). In all analyses, day was fitted as a covariate. To test whether patterns of conversion were nonlinear, each maximal model contained both linear and quadratic terms for day, but, in all models, all interactions and main effects for the quadratic were non-significant (\( p > 0.05 \)).

In our first analysis, we specified drug treatment as a fixed factor with three levels (chloroquine, pyrimethamine, control) and we specified whether parasites were resistant or sensitive as a fixed factor with two levels. Second, we tested whether the type of drug mattered by combining the chloroquine and pyrimethamine cultures and fitting drug treatment as a fixed factor with two levels (drugs, control). Third, we investigated whether our analysis could be simplified further by testing whether gametocyte conversion rates only differed between sensitive parasites in drug-treated cultures and all other groups that were either in control cultures or resistant to drugs. We fitted this as a fixed factor with two levels, by classifying parasites as safe (i.e. combining control cultures and resistant lines in drug-treated cultures) or vulnerable to drugs in their cultures (i.e. only sensitive lines in drug-treated cultures). By comparing the resulting minimal models from these three analyses, we obtained the most parsimonious description of conversion rates without significant loss of explanatory power. Finally, we tested whether the fixed effects providing the best description of conversion rates also influenced the densities of gametocytes and asexual stages observed during days 1–8 in the cultures.

3. RESULTS
Our first analysis revealed that the patterns of conversion rates observed during cultures were significantly influenced by the interaction between treatment, the drug sensitivity of parasites and day (\( \chi^2 = 6.49; \ p = 0.039 \)), but not by RBC density (\( \chi^2 = 0.96; \ p = 0.328 \)). Our second analysis, in which we collapsed the chloroquine- and pyrimethamine-treated groups into one factor level (table 1), also revealed that patterns of conversion rates
were significantly influenced by the interaction between treatment and whether parasites were sensitive or resistant ($\chi^2 = 5.81; p = 0.016$), but not by RBC density ($\chi^2 = 0.82; p = 0.365$). We compared the minimal models for our first and second analyses (AIC = 890.49 and 887.81, respectively), and found that there was no significant loss of deviance associated with the second, simpler model ($\chi^2 = 5.32; p = 0.256$), revealing that patterns of conversion rates did not significantly differ according to the type of drug parasites were treated with and that resistant parasites do not alter their conversion rates in response to drugs.

We then tested whether gametocyte conversion rates simply depended on whether parasites were both in drug-treated cultures and susceptible to drugs. This analysis revealed that parasites vulnerable to drugs in their cultures (the sensitive lines in drug-treated cultures) followed patterns with significantly lower conversion rates than parasites safe from drugs (figure 1; $\chi^2 = 23.74; p < 0.0001$). There was no additional influence of whether parasites were drug-sensitive or resistant ($\chi^2 = 0.34; p = 0.557$; interaction with day: $\chi^2 = 0.81; p = 0.367$), nor of RBC density ($\chi^2 = 0.002; p = 0.969$). Again, there was no significant loss of deviance when comparing the minimal models for our second and third analyses ($\chi^2 = 0.76; p = 0.944$; AIC = 887.81 and 880.57, respectively). This shows that conversion rates simply depend on whether parasites were vulnerable to, or safe from, drugs they were exposed to in their cultures.

In addition, we investigated whether the densities of gametocytes and asexual stages also differed according to whether parasites were vulnerable to, or safe from, drugs in their cultures. As expected, the lower conversion rates observed when parasites were vulnerable to drugs translated into the production of fewer gametocytes compared with parasites safe from drugs (figure 2a; $\chi^2 = 36.20; p < 0.0001$). In contrast, while the densities of asexual parasites varied during cultures ($\chi^2 = 164.11; p < 0.0001$), this was not significantly influenced by whether parasites were vulnerable to drugs (figure 2b; $\chi^2 = 1.47; p = 0.225$; interaction with day: $\chi^2 = 8.63; p = 0.374$). Moreover, the asexual densities of the sensitive lines did not differ significantly between treatments ($\chi^2 = 1.65; p = 0.199$).

4. DISCUSSION

We tested whether multiple lines of the human malaria parasite (*P. falciparum*) facultatively alter their allocation of resources to between-host transmission (gametocyte conversion rate) when exposed to low doses of different anti-malarial drugs. Our analyses show that gametocyte conversion rates were significantly lower in the drug-sensitive lines when exposed to drugs than for all parasites lines in drug-free control cultures, and than for the drug-resistant lines in drug-treated cultures (figure 1). Put simply, only the drug-sensitive lines decreased their investment into transmission stages when exposed to drugs. These data reveal that, as predicted, parasites adopt reproductive restraint when stressed by low doses of drugs. The drug-resistant lines did not significantly alter their conversion rates when exposed to drugs (and their conversion rates were not significantly different from the sensitive lines in control cultures), suggesting that parasites...
do not respond directly to drugs but instead adjust their conversion rates in response to changes in their proliferation (Koella & Antia 1995; Paul & Brey 2003). Information about their absolute density, or changes in density, could be obtained through a form of quorum sensing (Dyer & Day 1993) or the presence of lysed parasites, and other studies also suggest that parasites respond to their own density (Reece et al. 2008; Pollitt et al. submitted). Alternatively, as our lines were isolated at a time of frequent exposure to drugs, it is possible that the sensitive lines have evolved a specific response to these drugs, which the resistant parasites either never had or have subsequently lost. The latter could occur if there are costs associated with maintaining unnecessary information-gathering and processing systems (DeWitt et al. 1998). Investigating how parasites respond to drugs with different modes of action and different target stages could resolve this.

Our data reveal that parasites adopt reproductive restraint when exposed to low doses of anti-malarial drugs. This response is consistent with the hypothesis that reducing investment in gametocytes enables parasites to prioritize their survival by increasing their replication rate (Roff 1992; Stearns 1992; Koella & Antia 1995; Mideo & Day 2008; Fischer et al. 2009; McNamara et al. 2009). The asexual densities of sensitive strains were not significantly reduced in the drug-treated cultures, and we suggest that reproductive restraint enabled these parasites to reduce the detrimental effect of drugs. Quantifying the survival advantage gained by reducing investment in gametocytes requires experiments that measure the fitness consequences for parasites unable to respond to drugs, which in turn requires indentifying the mechanism parasites use to measure and respond to stress. This is also an area where mathematical modelling would be very useful. By calculating how much replication can be increased for a given reduction in gametocyte investment in gametocytes to in-host conditions may be constrained by how much the in-host environment can change during a cell cycle and during the maturation period of gametocytes. The cell cycle of rodent parasites is 24 h and gametocytes require less than 48 h to reach maturity, whereas P. falciparum parasites only replicate every 48 h and gametocytes require around 10 days to reach maturity (Garnham 1966). Rodent malaria parasites may be able to rapidly switch strategies, but P. falciparum parasites might be constrained to playing it safe and investing more in survival when in-host conditions are changing.

Third, our results may differ from previous studies with P. falciparum owing to variation in the ecology of the populations that isolates were collected from. Previous studies have used P. falciparum parasites from regions of high prevalence and year-round transmission, which are also well adapted to in vitro growth (Buckling et al. 1999b). Exposure to drugs may be a selection pressure that is too novel for these parasites to respond optimally to, and/or a fast drop in density may be used as a cue that survival is unlikely. In contrast, our lines have been maintained in an in vitro culture for only a limited time, and were isolated from an area with low and seasonal transmission, in which chronic infections are responsible for much of the malaria transmission and anti-malarial drug use is common (Babiker et al. 1991, 1998). Parasites in areas of high transmission may benefit from a ‘live fast, die young’ life history (Lines et al. 1991), but the value of future versus current reproduction is likely to be much higher for our parasites, and so they should be more likely to invest in survival when stressed.

Observations of changes in gametocyte carriage following drug treatment of natural infections in eastern Sudan—the region from which our parasites were isolated—are consistent with our results (Nassir et al. 2005; Ali et al. 2006). This issue is controversial because is not clear whether elevated gametocyte densities observed in natural infections after drug treatment are due to a change in investment or simply due to the release of sequestered gametocytes (Drakeley et al. 2006; Babiker et al. 2008). Our data suggest the latter, and if this occurs in natural infections, there will be implications for understanding and predicting the spread of drug resistance (Read & Huijben 2009). By reducing conversion rates in response to drug treatment, drug-sensitive parasites may transmit at a slower rate than drug-resistant parasites during exposure, but will be more likely to survive and, thus, transmit over a longer duration than currently expected. Determining whether the response of sensitive
parasites to drugs is a general response to stresses that influence their proliferation or a specific adaptation to drugs now is central to predicting the behaviour and evolutionary trajectories of parasites in populations with different exposure to drugs and transmission patterns. The opportunity to combine experiments with field data for the same population of human parasites offers a promising way to further the field of evolutionary medicine (Williams & Nesse 1991; Stearns & Koella 2007). Mathematical models, experiments and field data are now required to determine how parasite investment strategies influence the maintenance of drug-resistant parasites in natural populations, how their investment strategies vary with the magnitude and form of the stress they experience, and what the implications are for their virulence (Schneider et al. 2008). More generally, using an evolutionary approach to explain virulence hinges on the useful application of life-history trade-offs to parasites (Day 2003; Alizon et al. 2009). By revealing that malaria parasites trade off survival and reproduction in the manner explained by evolutionary theory for life histories, we can be more optimistic about understanding virulence.

We are grateful to the villagers of Asar village, eastern Sudan, for their cooperation during the course of the study, and the support of the Malaria Administration of the Sudanese Ministry of Health, together with the Malaria Control Department in Gedaref State and the staff of the Gedaref Hospital. We thank D. H. Nussey, L. C. Pollitt and N. Mideo for discussions, J. Koella and three anonymous reviewers for their comments and the Wellcome Trust and Medical Research Council, UK, for funding.

REFERENCES

Adjuik, M., Babiker, A., Garner, P., Olliaro, P., Taylor, W. & White, N. 2004 Artesunate combinations for treatment of malaria: metaanalysis. Lancet 363, 9–17.

Ali, E., Mackinnon, M. J., Abdel-Muhsin, A. B. A., Ahmed, S., Walliker, D. & Babiker, H. A. 2006 Increased density but not prevalence of gametocytes following drug treatment of Plasmodium falciparum. Trans. R. Soc. Trop. Med. Hyg. 100, 176–183. (doi:10.1017/S0031182005.04.021)

Alizon, S., Huford, A., Mideo, N. & van Baelen, M. 2009 Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. J. Evol. Biol. 22, 245–259. (doi:10.1111/j.1420-9101.2008.01658.x)

Auld, J. R., Agrawal, A. A. & Relyea, R. A. 2009 Re-evaluating the costs and limits of adaptive phenotypic plasticity. Proc. R. Soc. B 277, 503–511 (doi:10.1098/rspb.2009.1355)

Babiker, H. A., Creasey, A. M., Fenton, B., Bayoumi, R. A. L., Arnot, D. E. & Walliker, D. 1991 Genetic diversity of Plasmodium falciparum in a village in Eastern Sudan. I. Diversity of enzymes, 2D-PAGE proteins and antigens. Trans. R. Soc. Trop. Med. Hyg. 85, 572–577. (doi:10.1016/0035-9203(91)90347-2)

Babiker, H. A., Abdel-Muhsin, A. A., Ranford-Cartwright, L., Satti, G. & Walliker, D. 1998 Characteristics of Plasmodium falciparum parasites that survive the lengthy dry season in eastern Sudan where malaria transmission is markedly seasonal. Am. J. Trop. Med. Hyg. 59, 582–590.

Babiker, H. A., Schneider, P. & Reece, S. E. 2008 Gametocytes: insights gained during a decade of molecular monitoring. Trends Parasitol. 24, 525–530. (doi:10.1016/j.pt.2008.08.001)

Barnes, K. I. et al. 2007 World Antimalarial Resistance Network (WARN) IV: clinical pharmacology. Malaria J. 6, 122. (doi:10.1186/1475-2875-6-122)

Bayoumi, R. A. L., Creasey, A. M., Babiker, H. A., Carlton, J., Sultan, A. A., Satti, G., Sohal, A. K., Walliker, D. B. J. & Arnot, D. E. 1993 Drug response and genetic characterisation of Plasmodium falciparum clones recently isolated from a Sudanese village. Trans. R. Soc. Trop. Med. Hyg. 87, 454–458. (doi:10.1016/S0020-7519(93)90034-N)

Buckling, A. G. J. & Read, A. F. 1999 The effect of chloroquine treatment on the infectivity of Plasmodium chabaudi gametocytes. Int. J. Parasitol. 29, 619–626. (doi:10.1016/S0020-7519(98)00230-6)

Buckling, A. G. J., Taylor, L. H., Carlton, J. M. R. & Read, A. F. 1997 Adaptive changes in Plasmodium transmission strategies following chloroquine chemotherapy. Proc. R. Soc. Lond. B 264, 535–539. (doi:10.1098/rspb.1997.0079)

Buckling, A., Crooks, L. & Read, A. 1999a Plasmodium chabaudi: effect of antimalarial drugs on gametocytogenesis. Exp. Parasitol. 93, 45–54. (doi:10.1006/expar.1999.4429)

Buckling, A. G. J., Ranford-Cartwright, L., Miles, A. & Read, A. F. 1999b Chloroquine increases Plasmodium falciparum gametocytogenesis in vitro. Parasitology 118, 339–346. (doi:10.1017/S0031182099003960)

Carter, R. & Miller, L. H. 1979 Evidence for environmental modulation of gametocytogenesis in Plasmodium falciparum in continuous culture. Bull. World Health Organ. 57(Suppl. 1), 37–52.

Carter, R., Ranford-Cartwright, L. & Alano, P. 1993 The culture and preparation of gametocytes of Plasmodium falciparum for immunochemical, molecular, and mosquito infectivity studies. Methods Mol. Biol. 21, 67–88.

Day, T. 2003 Virulence evolution and the timing of disease life-history events. Trends Ecol. Evol. 18, 113–118. (doi:10.1016/S0169-5347(02)00049-6)

DeWitt, T. J., Sih, A. & Wilson, D. S. 1998 Costs and limits of phenotypic plasticity. Trends Ecol. Evol. 13, 77–81. (doi:10.1016/S0169-5347(97)01274-3)

Drakeley, C., Sutherland, C., Bousem, J. T., Sauerwein, R. W. & Targett, G. A. 2006 The epidemiology of Plasmodium falciparum gametocytes: weapons of mass dispersion. Trends Parasitol. 22, 424–430. (doi:10.1016/j.pt.2006.07.001)

Dyer, M. & Day, K. P. 1993 Regulation of the rate of asexual growth and commitment to sexual development by diffusible factors from in vitro cultures of Plasmodium falciparum. Am. J. Trop. Med. Hyg. 68, 403–409.

Ekvall, H., Premji, Z. & Bjorkman, A. 1998 Chloroquine treatment for uncomplicated childhood malaria in an area with drug resistance: early treatment failure aggravates anaemia. Trans. R. Soc. Trop. Med. Hyg. 92, 556–560. (doi:10.1016/S0035-9203(98)90093-0)

Fischer, B., Taborsky, B. & Dieckman, U. 2009 Unexpected patterns of plastic energy allocation in stochastic environments. Am. Nat. 173, E108–E120. (doi:10.1086/596536)

Garnham, P. C. C. 1966 Malaria parasites and other haemoparasites. Oxford, UK: Blackwell Science.

Jensen, J. B. & Trager, W. 1977 Plasmodium falciparum in culture—use of outdated erythrocytes and description of candle jar method. J. Parasitol. 63, 883–886. (doi:10.2307/3279900)

Koella, J. C. & Antia, R. 1995 Optimal pattern of replication and transmission for parasites with two stages in their life cycle. Theor. Popul. Biol. 47, 277–291. (doi:10.1016/tpbi.1995.1012)

Lines, J. D., Wilkes, T. J. & Lyimo, F. O. 1991 Human malaria infectiousness measured by age-specific sporozoite rates in Anopheles gambiae in Tanzania. Parasitology 102, 167–177.
Long, G. H., Chan, B. H. K., Allen, J. E., Read, A. F. & Graham, A. L. 2008 Blockade of TNF receptor 1 reduces disease severity but increases parasite transmission during *Plasmodium chabaudi* chabaudi infection. *Int. J. Parasitol.* 38, 1073–1081. (doi:10.1016/j.ijpara.2007.12.001)

McNamara, J. M. & Houston, A. I. 2009 Integrating function and mechanism. *Trends Ecol. Evol.* 24, 670–675. (doi:10.1016/j.tree.2009.05.011)

McNamara, J. M., Houston, A. I., Barta, Z., Scheurlein, A. & Fromhage, L. 2009 Deterioration, death and the evolution of reproductive restraint in late life. *Proc. R. Soc. B* 276, 4061–4066. (doi:10.1098/rspb.2009.0959)

Mideo, N., & Day, T. 2008 On the evolution of reproductive restraint in malaria. *Proc. R. Soc. B* 275, 1217–1224. (doi:10.1098/rspb.2007.1545)

Mideo, N., Day, T. & Read, A. F. 2008 Modelling malaria pathogenesis. *Cell. Microbiol.* 10, 1947–1955. (doi:10.1111/j.1462-5822.2008.01208.x)

Mutabingwa, T. K., Anthony, D., Heller, A., Hallett, R., Ahmed, J., Drakeley, C., Greenwood, B. M. & Whitty, C. J. M. 2005 Amodiaquine alone, amodiaquine + sulfadoxine pyrimethamine, amodiaquine + artesunate, and artemether-lumefantrine for outpatient treatment of malaria in Tanzanian children: a four-arm randomised effectiveness trial. *Lancet* 365, 1474–1480. (doi:10.1016/S0140-6736(05)66417-3)

Nassir, E., Abdel-Muhsin, A. M., Suliaman, S., Kenyon, F., Kheir, A., Geha, H., Ferguson, H. M., Walliker, D. & Babiker, H. A. 2005 Impact of genetic complexity on longevity and gametocytogenesis of *Plasmodium falciparum* during the dry and transmission-free season of eastern Sudan. *Int. J. Parasitol.* 35, 49–55. (doi:10.1016/j.ijpara.2004.10.014)

Paul, R. E. L. & Brey, P. T. 2003 Malaria parasites and red blood cells: from anaemia to transmission. *Mol. Cell.* 15, 139–149.

Paul, R. E. L., Coulson, T. N., Raibaud, A. & Brey, P. T. 2000 Sex determination in malaria parasites. *Science* 287, 128–131. (doi:10.1126/science.287.5450.128)

Paul, R. E. L., Ariei, F. & Robert, V. 2003 The evolutionary ecology of *Plasmodium*. *Ecol. Lett.* 6, 866–880. (doi:10.1046/1461-0248.2003.00509.x)

Peatey, C., Skinner-Adams, T., Dixon, M. W. A., McCarthy, J., Gardiner, D. & Trenholme, K. 2009 Effect of antimalarial drugs on *Plasmodium falciparum* gametocytes. *J. Inf. Dis.* 200, 1518–1521. (doi:10.1086/644645)

Pinheiro, J. C. & Bates, D. M. 2000 Mixed effects models in S and S-Plus. New York, NY: Springer Verlag.

Pollitt, L. C., Mideo, N., Drew, D. R., Colegrave, N., Schneider, P. & Reece, S. E. Submitted. Competition and the evolution of reproductive restraint in malaria parasites.

Puta, C. & Manyando, C. 1997 Enhanced gametocyte production in Fansidar-treated *Plasmodium falciparum* malaria patients: implications for malaria transmission control programmes. *Trop. Med. Int. Health* 2, 227–229. (doi:10.1046/j.1365-3156.1997.d01-267.x)

Read, A. F. & Huijben, S. 2009 Evolutionary biology and the avoidance of antimicrobial resistance. *Evol. Appl.* 2009, 40–51.

Read, A. F. & Taylor, L. H. 2001 The ecology of genetically diverse infections. *Science* 292, 1099–1102. (doi:10.1126/science.1059410)

Reece, S. E., Duncan, A. B., West, S. A. & Read, A. F. 2005 Host cell preference and variable transmission strategies in malaria parasites. *Proc. R. Soc. B* 272, 511–517. (doi:10.1098/rspb.2004.2972)

Reece, S. E., Drew, D. R. & Gardner, A. 2008 Sex ratio adjustment and kin discrimination in malaria parasites. *Nature* 455, 609–614. (doi:10.1038/nature06954)

Reece, S. E., Ramiro, R. S. & Nussey, D. H. 2009 Plastic parasites: sophisticated strategies for survival and reproduction? *Evol. Appl.* 2, 11–23.

Roff, D. A. 1992 *The evolution of life histories: theory and analysis*. New York, NY: Chapman and Hall.

Schlichting, C. D. & Pigliucci, M. 1998 *Phenotypic evolution: a reaction norm perspective*. Sunderland, MA: Sinauer.

Schneider, P., Chan, B. H. K., Reece, S. E. & Read, A. F. 2008 Does the drug sensitivity of malaria parasites depend on their virulence? *Malaria J.* 7, 257. (doi:10.1186/1475-2875-7-257)

Stearns, S. C. 1992 *The evolution of life histories*. Oxford, UK: Oxford University Press.

Stearns, S. C. & Koella, J. C. 2007 *Evolution in health and disease*. Oxford, UK: Oxford University Press.

Taylor, L. H. & Read, A. F. 1997 Why so few transmission stages? Reproductive restraint by malaria parasites. *Parasitol. Today* 13, 135–140. (doi:10.1016/S0169-4758(97)89810-9)

Trager, W. & Gill, G. S. 1992 Enhanced gametocyte formation in young erythrocytes by *Plasmodium falciparum* in vitro. *J. Protozool.* 39, 429–432.

Williams, G. C. 1966 *Adaptation and natural selection*. Princeton, NJ: Princeton University Press.

Williams, G. C. & Nesse, R. M. 1994 *The dawn of Darwinian medicine*. *Quarterly Review of Biology* 66, 1–22.