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Adaptive phenotypic plasticity in malaria parasites is not constrained by previous responses to environmental change

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ABSTRACT

Background and objectives: Phenotypic plasticity enables organisms to maximize fitness by matching trait values to different environments. Such adaptive phenotypic plasticity is exhibited by parasites, which experience frequent environmental changes during their life cycle, between individual hosts and also in within-host conditions experienced during infections. Life history theory predicts that the evolution of adaptive phenotypic plasticity is limited by costs and constraints, but tests of these concepts are scarce.

Methodology: Here, we induce phenotypic plasticity in malaria parasites to test whether mounting a plastic response to an environmental perturbation constrains subsequent plastic responses to further environmental change. Specifically, we perturb red blood cell resource availability to induce Plasmodium chabaudi to alter the trait values of several phenotypes underpinning within-host replication and between-host transmission. We then transfer parasites to unperturbed hosts to examine whether constraints govern the parasites’ ability to alter these phenotypes in response to their new in-host environment.

Results: Parasites alter trait values in response to the within-host environment they are exposed to. We do not detect negative consequences, for within-host replication or between-host transmission, of previously mounting a plastic response to a perturbed within-host environment.

Conclusions and implications: We suggest that malaria parasites are highly plastic and adapted to adjusting their phenotypes in response to the frequent changes in the within-host conditions they experience during infections. Our findings support the growing body of evidence that medical interventions, such as anti-parasite drugs, induce plastic responses that are adaptive and can facilitate the survival and potentially, drug resistance of parasites.

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INTRODUCTION

The ability to alter aspects of phenotype in response to changes in environmental conditions (phenotypic plasticity) is commonly observed in multicellular organisms [1–3]. But the notion that phenotypic plasticity is central to the fitness of parasites has been met with controversy in medicine and parasitology [4, 5]. This is likely due to the longstanding assumption that parasites, since they live inside their hosts, are sheltered from the rapidly changing conditions of the exterior environment, such as weather, temperature and predators, light and dark, and that there is a high autocorrelation in conditions experienced during infections and between hosts [6, 7]. An alternative view is that parasites are actually confronted with frequent and fast changing within-host conditions during each infection, as well as variation in conditions experienced in different hosts (particularly for parasites with complex, multi-host, life cycles). A number of within-host environmental factors vary during infections and across hosts, including the availability of resources, mating opportunities, immune responses, intensity of competition with co-infecting parasites, medical interventions and daily rhythms in host physiology. Parasites, stemming from diverse phyla, appear to cope with the challenges and opportunities arising from variable within-host conditions using phenotypic plasticity in a wide variety of traits. For example, the nematode *Strongyloides ratti* modifies its (generally stable) transcriptome in immunized hosts to avoid expulsion [8]. The trematode *Coitocaecum parvum* adopts a three-host or a two-host lifecycle depending on the availability of appropriate definitive hosts [9]. Bacteriophages vary their lysis/lysogeny decision, and malaria parasites their sex ratio and reproductive effort, in response to co-infecting conspecific genotypes [10, 11].

Like all traits, the evolution and expression of phenotypic plasticity is assumed to be subject to costs, constraints and trade-offs. These concepts are often hard to define and differentiate, and so are rarely examined empirically [12], especially for parasites/pathogens [5]. Costs of plasticity can be thought of as phenomena that reduce the fitness of a plastic genotype compared to a non-plastic genotype expressing the same trait value [12, 13]. Constraints of plasticity can be defined as phenomena that prevent organisms from reaching the optimal phenotype that a given environment demands [13]. For example, plasticity of many developmental traits is constrained by a window of sensitivity, i.e., a trait is only responsive to environmental change during early development and becomes fixed for the remaining lifetime of the individual. Thus, there is a risk that a trait is beneficial in early life but may become suboptimal at a later point because environmental conditions are likely to change. Such constraints include so-called ‘trans-host effects’ (in analogy with transgenerational effects on genetic expression), which occur when, for example, environmental conditions in the current host affect the behaviour of a parasite in the next host [14–17]. Analogous effects could occur during dynamic infections if parasites are unable to keep up with rapid changes in immune responses and resource availability. Thus, if parasites rely on phenotypic plasticity to optimize host exploitation and maximize transmission, then the history of hosts/cells/within-cell conditions that parasites have experienced could constrain the trait values of adaptive plastic responses to future conditions as well as reduce within-host replication, shorten infection duration or lower the rate of between-host transmission [18–20].

Here, we use the rodent malaria parasite *Plasmodium chabaudi* to explore the consequences of deploying plasticity. We do not test for inherent costs of plasticity because that would require comparing plastic and non-plastic parasites. Because non-plastic *P.chabaudi* genotypes do not exist, we use a different approach and test whether the effect of plasticity in response to an environmental change affects future responses and parasite performance. Malaria parasites replicate asexually in the vertebrate host’s red blood cells (RBC), but need to produce non-replicating sexual stages (‘gametocytes’) for between-host transmission, resulting in a trade-off between the allocation of resources to within-host replication and between-host transmission [21]. Malaria parasites adaptively adjust the proportion of each cohort of asexually replicating stages that commits to gametocyte development (‘conversion rate’) in response to within-host environmental conditions, including resource availability, competition with co-infecting parasites and antimalarial drug treatment [22–31]. Plasticity in other traits that underpin within-host replication also exists. For example, *Plasmodium berghei* reduces the number of progeny (merozoites) produced per asexual stage (‘burst size’) in response to caloric restriction of the host [32], and *P.chabaudi* increases burst size in anaemic hosts [33]. We harness the plastic responses of *P.chabaudi* to host anaemia [33] to investigate the impact of previous plastic responses on trait values for conversion rate, burst size and asexual replication. Specifically, we exposed parasites to either control hosts or anaemic hosts, to generate parasites with a different history of plasticity-driven trait values.
Subsequently, we mimic a rapid change of the within-host environment by transferring parasites from each control host and each anaemic host to non-anaemic hosts that serve as a common garden, i.e. parasites from both initial environments experience the same final within-host environment. An impact of the history of past within-host conditions and/or previously mounting a plastic response (beyond the reduction of asexual parasite densities), should manifest as a difference in trait values in the common garden hosts.

**METHODS**

The experiment comprised of two sets of hosts (Fig. 1). Parasites in initial hosts experienced a within-host environment of untreated hosts or of phenylhydrazine (PHZ) treated, anaemic hosts. Parasites were then transferred from the initial hosts to untreated, common garden hosts. We monitored the dynamics of asexual parasites and gametocytes, and burst size (number of merozoites/schizont) in the initial hosts to confirm parasites adopt different trait values due to mounting plastic responses to anaemia, and then monitored traits in the common garden hosts.

**Parasites and hosts**

We obtained C57BL/6 female mice (aged 6–8 weeks) in-house (University of Edinburgh) and the *P.chabaudi* clone AS (AS12537) from the Edinburgh Malaria reagent repository. All animal care was in accordance with institutional and UK Home Office guidelines. *P.chabaudi* was isolated from African thicket rats (*Grammomys poensis*, previously called *Thamommys rutilans*) in Central Africa [34], and *Mus musculus* is a natural host for some rodent malaria species [35]. After cloning, genotype AS has been cryopreserved and undergone regular transmission through mosquitoes to maintain its wild type phenotype [36]. We treated initial hosts 4 days before infection (day–4 post-infection (PI)) with a single intraperitoneal injection of either 30 mg/kg of PHZ dissolved in phosphate-buffered saline (PHZ, *n* = 14) or phosphate-buffered saline (Control, *n* = 11). PHZ causes anaemia by provoking the premature clearance of mature RBCs, leading to the release of immature RBCs (reticulocytes) into the blood as shown in [23] through an erythropoietin-mediated feedback loop [37].

All initial hosts received an intravenous injection of 5 × 10⁶ AS-parasitized RBCs on day 0 PI. On day 4 PI, we transferred 5 × 10⁶ parasitized RBCs from each initial host into a randomly chosen, untreated, common garden host (CG hosts, *n* = 25) by a one-to-one intravenous passage. Given that all hosts were inbred and age and sex matched, our design simulates a rapid change in within-host conditions (without the confounding effects of age of infection). We chose untreated hosts for our common garden hosts because *P.chabaudi* replicates faster in PHZ-treated compared to untreated hosts [23, 33]. This suggests that untreated hosts may provide a harsher environment for the parasites, in which constraints may be more easily observed. We designate the common garden hosts that received their parasites from a PHZ-treated initial host as an ‘ExPHZ’ infection (*n* = 14) and those that received their parasites from a control initial host as an ‘ExControl’ infection (*n* = 11). We monitored infections in the initial hosts from days 1 to 4 PI and from days 1 to 16 PI in the common garden hosts. Our design thus relies on three assumptions: first, that parasites encounter different within-host environments between PHZ-treated and control initial hosts, and second, that parasites exhibit different phenotypes between PHZ-treated and control initial hosts. Third, we assume that different phenotypes between the PHZ-treated and control initial hosts do not result from genetic adaptation to our reticulocyte-rich anaemic hosts. Such adaptation is extremely unlikely in our experiment for several non-mutually exclusive reasons: (i) parasites were expanded from cryopreservation in untreated hosts that are not reticulocyte-rich anaemic hosts. Any favourable variants would therefore have to be selected for in only three replication cycles in our initial hosts; (ii) for a single mutation to underpin such evolution, the highest possible estimate for a mutation to arise and expand in this time would result in the ‘adapted parasites’ to be at approx. 0.01% of the total parasite population; (iii) serial passage can result in genetic evolution in *P.chabaudi* but this generally requires 11–20 passages of infections each lasting at least 7 days [38–40]; (iv) plasticity may constrain genetic evolution because the ability to adjust trait values to suit the new environment buffers against fitness loss due to environmental changes, which in turn reduces the strength of selection on fixed traits [41]; (v) any adaptation to specifically infect reticulocytes is unlikely to have provided significant fitness benefits because even in PHZ-treated hosts, reticulocytes comprise a minority of the total RBC population.

**Data collection and analysis**

For all initial and common garden hosts, we measured RBC density and RBC age structure (the proportion of RBCs that are immature RBCs; i.e. reticulocytes), the proportion of RBCs infected with asexual stages and gametocyte density daily, with the exception of day 1 PI gametocyte density in the initial hosts. We quantified RBC density from 2 μl blood by flow cytometry (Beckman Coulter), and made thin blood smears to count the ratio of mature (normocytes) to immature (reticulocytes) RBCs, as well as the proportion of asexual stage infected RBCs, by microscopy. We quantified gametocyte densities from 10 μl blood samples by reverse-transcriptase quantitative PCR [42] as described previously [23]. We assessed burst size on day 4 PI as the number of merozoites counted from the first 30 mature schizonts observed on blood smears taken shortly before schizogony (from a random subset of *n* = 4 per treatment for the initial hosts, and for all common garden host infections).
We characterized the within-host environment based on the density of total RBC (i.e. the combined density of normocytes and reticulocytes) and their age structure (i.e. the proportion of RBCs that are reticulocytes). We calculated daily asexual parasite densities by multiplying RBC density with the proportion of infected RBCs, and used daily asexual parasite densities to derive replication rate (replication rate day \( t = \frac{\text{asexual parasite density day } (t+1)}{\text{asexual parasite density day } (t)} \)). It was only possible to calculate conversion rates for infections of CG hosts since data from a longer infection duration than in the initial hosts is required to use a state-of-the-art spline-based model, developed by Greischar et al. [43].

All calculations and statistical analysis were carried out using R version 3.2.4. To analyse the dynamics of host and parasite traits during the infections, we used linear mixed effect models including treatment, day PI and their interaction as fixed effects, and mouse identification number as a random effect to account for repeated measures. For the analyses of burst size data we used linear mixed effect models including treatment as a fixed effect, and mouse identification number as a random effect to account for repeated measures. We minimized nested models using maximum likelihood deletion tests, and we transformed response variables to meet assumptions of homogeneity of variance (proportion of reticulocytes and replication rates were square root transformed, whilst asexual parasite density, gametocyte density and conversion rate were log_{10} transformed adding half a counting unit to all data to deal with zero values according to [44]). To compare infections in initial with common garden hosts, we used only data covering the same sample time points in both data sets. We first used the above approach to verify that there was a difference between the four host types, and subsequently compared the four host type model with a model that groups untreated initial hosts with the common garden hosts (i.e. PHZ vs Control+ExControl+ExPHZ) to confirm that the PHZ is the outlying group. We present significant test statistics in the text, whilst full statistical details are shown in Supplementary Table S1.

**RESULTS**

Our experiment required comparing plasticity in parasite traits for parasites originating from either untreated (Control) or anaemic, PHZ-treated initial hosts, after being placed in the same environment: untreated, non-anaemic common garden hosts (ExControl, ExPHZ). First, we confirm the assumption that parasites experience different environments in Control and PHZ-treated initial hosts. Second, we confirm that parasites adopt different phenotypes in Control and PHZ-treated initial hosts. Third, we compare the trait values of parasites—stemming from different types of initial hosts—in common garden hosts (ExControl vs ExPHZ), to test for effects of previous plasticity, i.e. the trait values adopted in initial hosts.

**RBC environments and parasite traits vary between PHZ-treated and control initial hosts**

PHZ treatment generated anaemia, which was maintained in the PHZ-treated initial hosts throughout days 0–4 PI, as measured by a decrease in RBC densities (treatment * dayPI interaction: \( \chi^2 (4) = 13.92, P = 0.0076 \)) and an increased proportion of RBCs that were reticulocytes (treatment effect: \( \chi^2 (1) = 44.31, P < 0.0001 \)) (Fig. 2A and B).
Parasites adopted different trait values in control and PHZ-treated initial hosts in the manners described previously [23, 33]. First, daily asexual replication rates varied between treatments (treatment * dayPI interaction: $\chi^2(2) = 15.96, P = 0.0003$), with replication rates in PHZ-treated hosts higher than in untreated hosts on day 1 PI (Fig. 2C). This results in differences between treatments for the dynamics of asexual densities during days 1–4 PI (treatment * dayPI interaction: $\chi^2(3) = 21.51, P < 0.0001$), with higher asexual parasite densities in PHZ-treated initial hosts than in control initial hosts during days 2–4 PI (Fig. 2D). Second, parasites in PHZ-treated initial hosts produced, on average, 1.15 times more merozoites per schizont than parasites in control initial hosts ($\chi^2(1) = 12.73, P = 0.0004$, Fig. 2E). Third, the dynamics of gametocytes densities differed between treatments (treatment * dayPI interaction: $\chi^2(2) = 9.92, P = 0.0070$), with gametocyte densities in PHZ-treated initial hosts higher than those in control initial hosts (Fig. 2F).

**Trait values in the common garden hosts**

The dynamics of RBC densities (treatment: $\chi^2(1) = 0.002, P = 0.9617$) and of proportion of reticulocytes (treatment: $\chi^2(1) = 0.001, P = 0.9719$) were not significantly different between the two groups of untreated common garden hosts (ExPHZ and ExControl) for days 0–16 PI (Fig. 3A and B). For the time frame during which data were collected in the initial hosts (days 0–4 PI), the RBC environment did not significantly differ between the untreated common garden hosts (ExPHZ and ExControl) and the control initial hosts (RBC density $\chi^2(10) = 9.74, P = 0.4634$; proportion reticulocytes $\chi^2(10) = 12.07, P = 0.2804$). Thus, the assumption that parasites in control initial hosts and all common garden hosts experienced the same RBC environment was met. We did not detect any significant differences in asexual stage traits between ExPHZ and ExControl parasites (Fig. 3C–E). This includes the dynamics of replication rate (Fig. 3C, treatment: $\chi^2(1) = 0.27, P = 0.6059$) and of asexual densities (Fig. 3D, treatment: $\chi^2(1) = 0.03, P = 0.8625$), and day 4 PI burst sizes (Fig. 3E, treatment: $\chi^2(1) = 0.058, P = 0.8103$). We did observe a difference in gametocyte dynamics between ExPHZ and ExControl parasites (Fig. 3F, treatment * dayPI interaction: $\chi^2(15) = 45.24, P < 0.0001$), driven by gametocyte densities at the start of infections. Gametocytes on day 1 PI are likely carried over from the initial hosts as well as originating from the conversion decision of...
the asexual parasites in the inoculum used to infect the common garden hosts, and therefore reflect the RBC environments of the initial hosts rather than reflecting the performance of parasites in common garden hosts. When excluding day 1 PI from the analysis, neither the treatment × dayPI interaction ($\chi^2 (14) = 13.79, P = 0.4654$) or treatment ($\chi^2 (1) = 0.0008, P = 0.977$) were significant (Fig. 3F). Furthermore, daily conversion rates, i.e. the proportional investment of asexual parasites into gametocytes, were not significantly different between ExPHZ and ExControl parasites (Fig. 3G, treatment: $\chi^2 (1) = 0.18, P = 0.6748$).

**DISCUSSION**

We asked whether mounting phenotypically plastic responses to a perturbation of the within host environment imposes longer-term constraints on future plastic responses. Harnessing the diverse phenotypically plastic traits expressed by *P.chabaudi* malaria parasites in response to host anaemia revealed no such effects. Specifically, parasites transferred from anaemic initial hosts exhibited trait values that were not significantly different to those of parasites transferred from control initial hosts. We assume that plasticity in the traits investigated here are at least in part under parasites transferred from control initial hosts. We did not directly quantify conversion rates in the initial hosts, this implies conversion rates were increased in PHZ-treated hosts, as shown previously [23]. Any costs/constraints related to such elevated conversion rates did not affect the replicative capacity of asexual stages or future conversion to gametocytes in common garden hosts. Whilst conversion rate is the plastically adjusted trait, the resulting density of gametocytes is better proxy for short-term transmission probability (i.e. parasite fitness). We found no evidence of fitness costs in terms of lower gametocyte densities in parasites stemming from anaemic initial hosts.

The absence of a detectable effect of previous plasticity may have several, non-mutually exclusive, explanations. (i) It is possible that our experimental design was unable to detect small effects due to low power. (ii) Parasites may readily change reticulocyte/normocyte invasion ligands upon arrival in the common garden hosts. (iii) The repertoire of invasion ligands expressed by *P.chabaudi* may always be diverse enough for efficient invasion independent of RBC age structure [33]. (iv) If asexual density is viewed as a fitness proxy, the lower density in control initial hosts on the day of transfer could be viewed as a cost, but this was ameliorated by initiating infections in common garden hosts with a fixed number of parasites. This approach controls the impact of inoculum dose on infection dynamics, allowing us to test for effects of ‘quality’ rather than ‘quantity’ of parasites. (v) Costs/constraints may only manifest as trans-host, or host-vector effects, which we have not tested for. Although we use a sequence of hosts (initial and common garden hosts), we transferred parasites by blood passage, rather than by mosquito (which is the natural route for transmission), to simulate the changes in RBC density and age structure experienced during infections. (vi) The common garden environment provided by naïve hosts was not stressful enough to elicit constraints. For example, Steinger et al. report that sib-families of the plant *Sinapis arvensis* that show greater plasticity in specific leaf area also suffer larger fitness costs in a shady environment than in full light conditions [49], and plasticity in internode length in *Ranunculus reptans* is more costly in competitive than under benign conditions [50]. Intuitively, naïve hosts appear to be a benign environment. However, given that parasites replicate faster in PHZ-treated compared to untreated hosts [23, 33], this suggests that untreated hosts are the harsher environment, but perhaps not harsh enough. (vii) We tested a single parasite genotype to balance ethical considerations with testing for proof of principle. It is possible that our results may not be easily generalized to other genotypes. However, we generally see qualitatively similar responses to environmental perturbations across the available *P.chabaudi* genotypes. Genotype by environment interactions are sometimes observed but these result in quantitative, rather than qualitative, differences in the genotypes’ responses to environmental change (e.g. [11, 23, 25, 33]). Further, across the *P.chabaudi* clone bank, the genotype we chose (AS) displays an intermediate level of plasticity in the traits we studied (a.o. [23]), so we expect it is representative of the population. (viii) We only exposed parasites to a single environmental change (anaemic to control hosts) and constraints and limits may only emerge after parasites experience a longer sequence of different within-host conditions. If costs and constraints are mainly
exposed in stressful environments [51], future work could examine parasites in common garden hosts that involve competition with con-specific genotypes, in semi-immune hosts or during drug treatment.

One of the preconditions for plasticity to evolve is that its costs, relative to the negative consequences of suboptimal trait values, must be low [12, 13]. For parasites that live in variable environments or that require extensive changes to their traits, selection may have honed rapid responses and minimized costs/constraints. For example, the parasitic nematode *Heligmosomoides polygyrus* increases expression of two immunomodulatory genes in hosts mounting an inflammatory response, but readily reduces expression when in hosts without inflammatory responses, even after experiencing upregulated inflammation for four parasite generations [52]. If costs and constraints do exist, they may only be exposed in stressful environments [51]. Thus future work could examine parasites in common garden hosts that involve competition with con-specific genotypes, in semi-immune hosts, or during drug treatment. By using a single parasite genotype (to avoid any confounding effects of genetic variation), we assume that conversion rates vary day-to-day (Fig. 3G), parasites in both types of initial host and in common garden hosts are paying the costs of environmental sensing. The standing costs of plasticity are thought to be low, but are hard to measure [12, 13]; future

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**Figure 3.** The within-host environment and parasite traits do not differ between common garden hosts (ExPHZ, green and ExControl, purple). We observed no significant differences in RBC densities (RBC dens, A), the proportion of RBCs that are reticulocytes (Prop retics, B), asexual replication rates (C), asexual parasite densities (Asex dens, D) or burst sizes, measured as the number of merozoites per schizont, on day 4 PI (E) between ExPHZ and ExControl hosts. Gametocyte densities are higher in ExPHZ compared to ExControl hosts on day 1 PI only because these gametocytes originate from infections in the initial hosts (Gct dens, F) and conversion rates were not significantly different between common garden hosts (G). Graphs show mean ± SEM (A–D, F–G) or median (black dashed) and 25–75 percentiles, with whiskers 1.5 times the interquartile range and dots representing outliers (E).
work that selects for parasites with a fixed conversion rate might allow these costs to be measured. Finally, the generality of our results can be confirmed by extending experiments to include multiple genotypes and species of malaria parasites.

Here we show that parasites are able to rapidly adjust fitness-related life history traits to match changes in conditions experienced in the within-host environment. Mechanisms controlling environmental sensing in malaria parasites are only partly understood (e.g. [32]), but evidence for epigenetic mechanisms governing commitment to gametocytes is accumulating rapidly [53–56]. Thus, in the context of conversion at least, our findings suggest these epigenetic mechanisms quickly respond to environmental change. The capacity for rapid and generally appropriate responses to environmental change may play an underappreciated role in treatment failure and the evolution of resistance [41]. For example, plasticity in conversion allows parasites to find the best balance between short- and long-term transmission after drug treatment [28]. Given that different types of environmental change demand different trait values, could parasites be forced to make suboptimal decisions by exposing them to several such environmental changes, simultaneously?

**SUPPLEMENTARY DATA**

Supplementary data is available at EMPH online.

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