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Turning up the heat: heat stress induces markers of programmed cell death in *Plasmodium falciparum in vitro*

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Malaria is characterised by cyclical febrile episodes that result from the rupture of mature schizont-infected erythrocytes releasing merozoites. In patients infected with *Plasmodium falciparum*, fever may reach peak temperatures as high as 41 °C. Febrile episodes typically have a deleterious effect on parasites and probably benefit the host by aiding parasite clearance; however, the parasite may also gain advantage from limiting its burden on the host and prolonging infection to ensure development and transmission of slow-maturing gametocytes. Programmed cell death (PCD) may provide the parasite with a mechanism of self-limitation, although the occurrence and phenotype of PCD in the erythrocytic stages remain controversial due to conflicting data. This study aimed to characterise the cell death phenotype of *P. falciparum* in response to *in vitro* heat stress. A variety of biochemical markers of PCD, including DNA fragmentation, mitochondrial dysregulation and phosphatidylserine externalisation, as well as morphological studies of Giemsa-stained thin smears and real-time microscopy were utilised to characterise the phenotype. Heat stress decreased *P. falciparum* growth and development *in vitro*. Late-stage parasites were more susceptible, although early stages were more affected than expected. Early-stage parasites exposed to 41 °C exhibited markers of an apoptosis-like PCD phenotype, including DNA fragmentation and mitochondrial depolarisation. Heat-stressed late-stage parasites showed no significant DNA fragmentation or mitochondrial dysregulation; however, cytoplasmic vacuolisation was suggestive of an autophagy-like form of PCD. Our results therefore showed that biochemical and morphological markers of PCD varied with intra-erythrocytic parasite development and that *P. falciparum* exhibited facets of both apoptosis- and autophagy-like phenotypes after exposure to febrile temperatures, which may reflect a unique PCD phenotype.
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Malaria is characterised by acute cyclical febrile episodes that are triggered by the rupturing of mature schizont-infected erythrocytes releasing merozoites.[1] In *Plasmodium falciparum* malaria, fever typically recurs every 48 h,[2,3] coinciding with the parasite's erythrocytic life cycle,[2] and may peak as high as 41 °C for 2–6 h.[3] However, multiple asynchronous infections may lack periodicity[3] and febrile episodes may therefore occur more frequently and last longer.

Elevated temperatures of 40 °C for 6 h or more significantly inhibited *P. falciparum* growth *in vitro*,[4–6] with greater than 95% parasite death after 48 h.[6] Exposure to 41 °C significantly decreased parasite survival after only 2 h,[7] with parasite death noticed after as little as 30 min exposure.[8] The late developmental stages of trophozoites and schizonts were more susceptible to hyperthermal damage, whereas the ring stages were more resistant,[4–8] with exposures to 40 °C causing synchronization towards the ring stage.[4,8] Exposure to 41 °C

has been shown to also inhibit the growth of these early stages.[7,8] Although febrile episodes are generally considered to benefit the host by suppressing parasite growth and assisting in clearance,[5] parasites may also derive benefit from increased temperatures. *In vitro* studies have shown that *P. falciparum* growth rates and development were accelerated when parasites were exposed to 40 °C for 2 h, incubated at 37 °C for 10 h and again exposed to 40 °C for 12–24 h.[9] The parasite life cycle was also accelerated after exposure to less severe temperatures of 38.5–39 °C.[10] Incubation at 40 °C for 1–2 h enhanced the cytoadherence of mature *P. falciparum*-infected erythrocytes (pRBC) to CD36 and ICAM-1 receptors and also caused adherence of ring-stage parasites, which do not normally bind to these receptors.[11] Febrile temperatures have also been shown to reduce the deformability of pRBC, which may aid in sequestration,[12] as well inducing phosphatidylserine (PS) externalisation in pRBC.[10]

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Heat shock triggers activation of the intrinsic apoptosis pathway in metazoans, resulting in permeabilisation of the mitochondrial outer membrane with concurrent depolarisation of the mitochondrial transmembrane potential ($\Delta\Psi m$) and the initiation of an enzyme cascade that ends in DNA fragmentation.[13] A growing body of evidence suggests that *P. falciparum* may undergo programmed cell death (PCD), although some conflicting results have suggested a range of cell death phenotypes, including apoptosis, autophagy-like cell death, necrosis or simply undetermined. Phenotypes may also not be exclusive and overlap may occur.[14] According to the best of our knowledge, data regarding the possible induction of PCD by heat stress are limited to two studies, which have offered conflicting conclusions, with an apoptosis-like form of PCD suggested on the one hand[7] and the other suggesting that cell death more closely resembled secondary necrosis, although some form of PCD was not ruled out.[6]

We present a comprehensive *in vitro* study that utilised a variety of biochemical and morphological markers of cell death, as well as heat stress of different duration and intensity, to provide extensive characterisation of the response of *P. falciparum* to conditions similar to febrile episodes experienced during malaria. *P. falciparum* exhibited markers of PCD, including DNA fragmentation, mitochondrial dysregulation, PS externalisation and cytoplasmic vacuolisation. However, early and late intra-erythrocytic stages differed in their response to heat stress and exhibited different phenotypes, which may represent different facets of a single PCD mechanism unique to *P. falciparum*. Febrile temperatures may induce self-limitation of parasite populations through PCD *in vivo* to the benefit of both the host and parasite. Elucidation of a PCD mechanism distinct from metazoans in *P. falciparum* may yield novel drug targets to be exploited in manipulating parasite fate.

Results

Heat stress inhibits *P. falciparum* growth and development.
Parasitised cultures were exposed to heat stress to mimic either the extended fever periods experienced during prolonged malaria (40 °C for 6 or 24 h, Figure 1) or the occasional high peaks of fever paroxysms (41 °C for 2 h, Figure 2), and the response of the parasites was characterised with biochemical markers of PCD (Table 1). We observed time-dependant inhibition of growth and development by exposure to 40 °C, with late-stage parasites more severely affected. After 6 h exposure, an apparent delay was noted in the development of ring-stage parasites to late stages between 24 and 48 h (Figure 1). Ring-stage parasites became more vulnerable to heat stress at 41 °C and were more affected than previously thought: ~ 75% of early-stage parasites exposed to 41 °C for 2 h (Figure 2) failed to develop, compared with a 25% reduction in parasite survival previously shown under the same conditions.[7] The effect of heat stress on late-stage parasites also became more pronounced at 41 °C, with very few parasites observed 24 h after exposure, in comparison with control parasites maintained at 37 °C, which replicated to form new rings (Figure 2).

Heat stress induces markers of PCD in *P. falciparum*.
P. falciparum exposed to heat stress exhibited a number of markers of PCD, including DNA fragmentation, mitochondrial dysregulation, PS externalisation and cytoplasmic vacuolisation, which varied with the developmental stage and in some instances with the temperature and duration of heat stress (see Table 1 for summary).

DNA fragmentation, quantified by the flow cytometric TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay, was observed in mixed-stage cultures exposed to 40 °C for 24 h (Figures 3aii and bii) but not for 6 h (Figures 3ai and bi). Therefore, 24 h was chosen for further experiments involving exposure to 40 °C. DNA fragmentation was also observed in synchronised ring-stage parasites exposed to 41 °C for 2 h (Figures 3aiii and biii). Surprisingly, late-stage parasites exposed to the same stress exhibited insignificant DNA fragmentation (Figures 3aiv and biv) at 24 h, despite a significant decrease in parasitaemia (Figure 2). Therefore, exposure to 41 °C may have caused the formation of nonviable merozoites that failed to invade new cells, whereas surviving parasites likely sustained little or no damage.

Mitochondrial depolarisation, generally considered to be characteristic of apoptosis,[15–20] was observed immediately following heat stress in early-stage parasites exposed to 40 °C for 24 h (Figures 4ai and bi), although it was not noted the following day (Figures 4aii and bii). Early-stage parasites exposed to 41 °C for 2 h exhibited slight mitochondrial depolarisation immediately after heat stress (Figures 4aiii and biii) that was significant the next day (Figures 4aiv and biv). Many of the ring-stage parasites exposed to 40 °C developed further (Figure 1), whereas ~75% of ring-stage parasites exposed to 41 °C did not (Figure 2aiii). Surprisingly, late-stage parasites exposed to 40 °C for 24 h exhibited mitochondrial hyperpolarisation (Figures 5ai and bi), that was less prominent the following day (Figures 5aii and bii). No change was observed in the $\Delta\Psi m$ of late-stage parasites exposed to 41 °C (Figures 5aiii-iv and biii-iv). In accordance with previous studies,[10] increased PS externalisation was observed in late-stage pRBC exposed to 41 °C a day after heat stress was withdrawn (Figures 6avi and bvi). No increase was noted in synchronised ring-stage pRBC immediately after exposure to 41 °C for 2 h (Figures 6aiii and biii), with an apparent decrease noted at 48 h (Figures 6aiv and biv); however, when comparing the heat-stressed culture at both time points (iii and iv), no change was apparent. PS externalisation was also noted in mixed cultures immediately after 24 h exposure to 40 °C (Figures 6ai and bi).

Most late-stage parasites exposed to 41 °C for 2 h were observed outside erythrocytes on Giemsa-stained thin smears at 24 h, whereas many intracellular parasites exhibited jagged borders and prominent vacuoles within the cytoplasm (Figure 7). Real-time microscopy showed that many of the remaining intracellular parasites, despite not replicating to new ring stages, presented with continued rapid movement (Supplementary Videos) similar to that observed in 37 °C controls and considered characteristic of healthy parasites.[21] A clear recovery, evidenced by increased parasitaemia (Supplementary Figure 1), was noted 5 days after heat stress. Ring-stage parasites exposed to the same
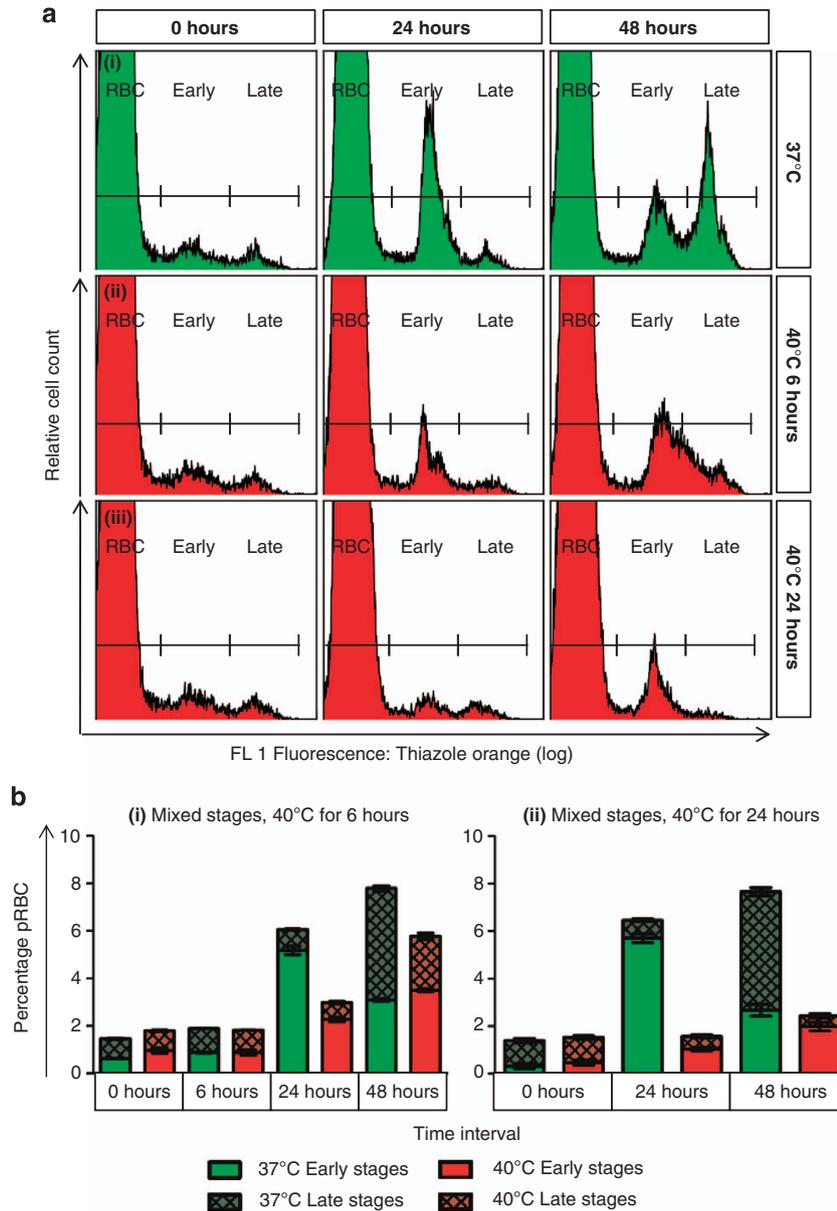
Figure 1 Exposure to 40 °C inhibited growth and development in mixed-stage *P. falciparum* in an exposure-dependant manner. Compared with control parasites kept at 37 °C (**ai**), cultures exposed to 40 °C for 6 or 24 h (**aii** and **iii**, respectively) showed an exposure-dependant inhibition in the formation of new ring-stage parasites (Early) from late-stage parasites (Late) at 24 h, with a correspondingly smaller late-stage population at 48 h (**b**). Late-stage parasites were more affected by heat stress. However, a delay in the development of ring-stage parasites to late stages was apparent between 24 and 48 h after 6 h exposure, which resulted in a broad, indiscrete peak at 48 h (**aii**), indicating a population of intermediate development. Furthermore, after 24 h exposure, few early-stage parasites at 24 h appeared to progress to late stages at 48 h (**aiii** and **bii**). Raw flow cytometry histograms (**a**) and stacked bar graphs (**b**) are shown. Stacked bar graphs show changes in parasitaemia (total bar height) and relative composition of the parasite population with respect to early- and late-stage parasites. For (**bi**) $n = 2$, for (**bii**) $n = 6$ at 24 h and $n = 4$ at 48 h. Data points represent arithmetic mean ± S.E.M. Heat stress was applied at 0 h

stress conditions also showed abnormal morphology on Giemsa-stained thin smears (Figure 7), although morphologically normal parasites were also observed.

Discussion

In vitro P. falciparum exhibited various biochemical markers of PCD, including DNA fragmentation, mitochondrial dysregulation and PS externalisation, as well as some abnormal morphological features including cytoplasmic vacuolisation, in response to heat stress. Different stages of the parasite life cycle exhibited a different combination of PCD markers, which also varied depending on the temperature and the duration of the heat stress.

Heat stress in *P. falciparum*: a single degree makes a difference. Several previous studies have shown that febrile temperatures inhibit *P. falciparum* growth *in vitro*[4,5,8,22] and
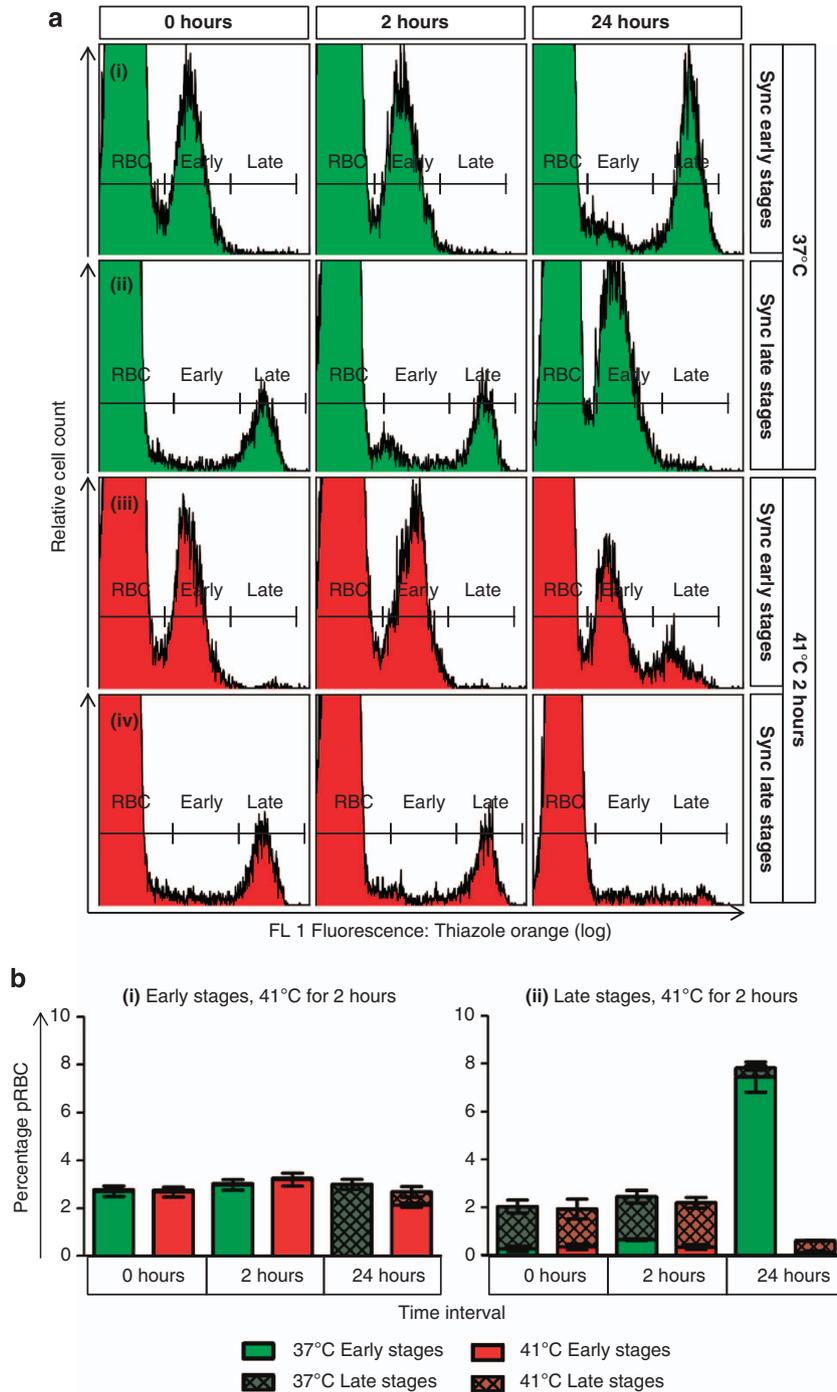
Figure 2 Exposure to 41 °C for 2 h inhibited replication and development of both early- and late-stage synchronised *P. falciparum*. Representative flow cytometry histograms (**a**) showed the progression of early (**i**) and late (**ii**) stage parasites kept at 37 °C, in comparison with early (**iii**) and late (**iv**) stage parasites exposed to 41 °C for 2 h. Time intervals are indicated on top, whereas synchronisation and treatment conditions are indicated on the right. (**b**) Stacked bar graphs show changes in parasitaemia (total bar height) and relative composition of the parasite population with respect to early- and late-stage parasites. A small portion of ring stage (Early) parasites exposed to 41 °C developed to late stages (Late), while most remained as ring-stage parasites. Late stages exposed to 41 °C failed to produce viable merozoites to infect erythrocytes and virtually no parasites were detected at 24 h, compared with 37 °C control parasites, which showed a large new ring population. Data points represent arithmetic mean ± S.E.M. ($n = 4$ for **bi** and $n = 5$ for **bii**). Heat stress was applied at 0 h

have noted that the effect varies with temperature.[5,8] However, only two studies have investigated the appearance of PCD markers in response to heat stress and those studies utilised different strains, cell death markers and heat stress conditions, and have offered conflicting conclusions.[6,7] We utilised heat stress conditions similar to both previous studies and scored a variety of biochemical and morphological markers to characterise the cell death phenotype in

Table 1 Summary of biochemical markers of cell death observed in *P. falciparum* after exposure to increased temperatures

Parasite life stage	Early stages at 0 h		Late stages at 0 h	
Exposure conditions	40 °C for 24 h	41 °C for 2 h		
Time of measurements	Immediately after heat stress	22 h after heat stress	Immediately after heat stress	22 h after heat stress
Growth and development	Slight growth inhibition. Progression to late stages was similar to 37 °C control parasites (Figure 1)	Inhibition of growth. A small number developed to late stages (Figure 2)	Severe growth inhibition with very few ring parasites observed (Figure 1)	>95% growth inhibition, with no new ring-stage parasites formed (Figure 2)
DNA fragmentation	*	Yes (Figure 3aiii)	*	Slight (Figure 3aiv)
Mitochondrial polarisation	Depolarised (Figure 4bi)	Depolarised (Figure 4bii)	Hyperpolarised (Figure 5bi)	No change (Figure 5bii)
PS externalisation	*	No (Figures 6c and d)	*	Yes (Figure 6f)

Parasite life stages, incubation conditions and relevant figures for each result are indicated. Asterisks (*) indicate no data, as effects were observed in mixed-stage cultures
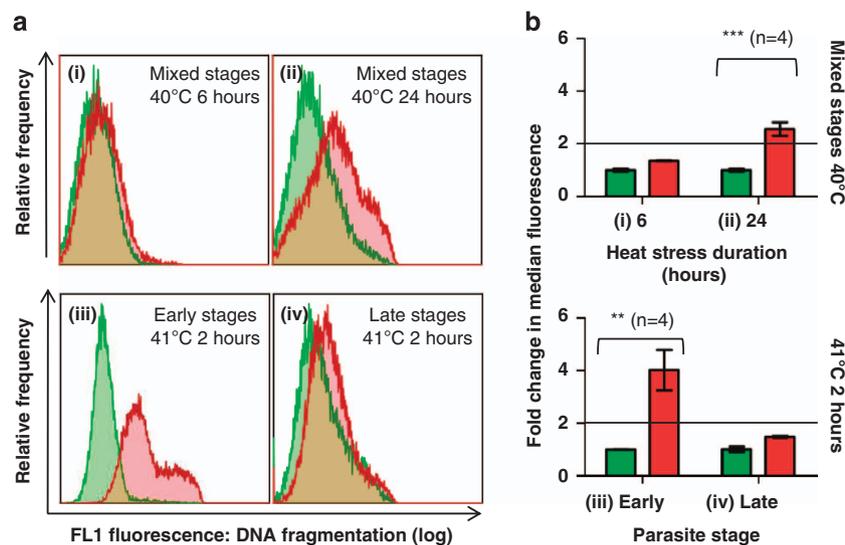

Figure 3 Heat stress induced DNA fragmentation in *P. falciparum*, as measured by the TUNEL assay. Control parasites, incubated at 37 °C, are indicated in green, whereas heat-stressed parasites are indicated in red. All samples were collected at 24 h. TUNEL results indicated significant DNA fragmentation in mixed-stage parasites exposed to 40 °C for 24 h and synchronised ring-stage parasites exposed to 41 °C for 2 h, with only slight fragmentation in synchronised late-stage parasites exposed to the same stress, compared with control parasites at identical time intervals. (**a**) Overlaid fluorescence histograms of the TUNEL assay indicate the FL1 (dUTP-FITC) fluorescence of isolated parasites from individual heat-stressed (red) or control (green) data sets. Fragmentation resulted in a higher fluorescence, indicated by a shift towards the right. Heat stress conditions are indicated in the top right corner of each overlay. Figure numbers (**i–iv**) match those of the bar graphs. (**b**) Bar graphs summarise TUNEL results for mixed-stage parasites exposed to 40 °C for 6 (**i**) and 24 (**ii**) hours, as well as synchronised early (**iii**) and late (**iv**) stage parasites exposed to 41 °C for 2 h. Changes in fragmentation are indicated as the mean change in median fluorescence for all samples. Differences of twofold or greater (indicated by a horizontal line on bar graphs) between heat-stressed and corresponding 37 °C control parasites are considered indicative of fragmentation. Data points indicate the arithmetic mean of replicates ± S.E.M. Comparisons between control and heat-stressed parasites showed *P*-values that are significant at <0.01 (**) or <0.001 (***)

intra-erythrocytic *P. falciparum* after heat stress. An apoptosis-like phenotype was previously suggested based on gene-expression studies in asynchronous parasites and DNA fragmentation detected in schizont-stage parasites by the *in situ* TUNEL assay, after exposure to 41 °C for 2 h.[7] After the same exposure, our data showed no significant DNA fragmentation in late-stage parasites; however, we did observe DNA fragmentation in ring-stage parasites, which Oakley *et al.*[7] did not consider. We also utilised flow cytometry to quantify the TUNEL assay, rather than fluorescence microscopy.[7] After exposure to 40 °C for various durations up to 48 h, Porter *et al.*[6] reported a lack of DNA fragmentation or caspase activation, mitochondrial depolarisation in late-stage parasites as well as food vacuole

swelling and lysis. Although heat-induced PCD was not ruled out, it was suggested that the parasite cell death response more closely resembled secondary necrosis.[6] In contrast, we observed significant DNA fragmentation in mixed-stage cultures after exposure to 40 °C for 24 h, quantified by the TUNEL assay, which is more sensitive than the agarose gels utilised previously.[6] Furthermore, we surprisingly found mitochondrial hyperpolarisation in late-stage parasites, whereas only early-stage parasites showed depolarisation. It is worth noting that, although we utilised similar mitochondrial staining methods, we discriminated between early- and late-stage pRBC based on hydroethidine (HE) uptake, whereas Porter *et al.*[6] used synchronised cultures. Our data showed that differences in heat stress conditions may at
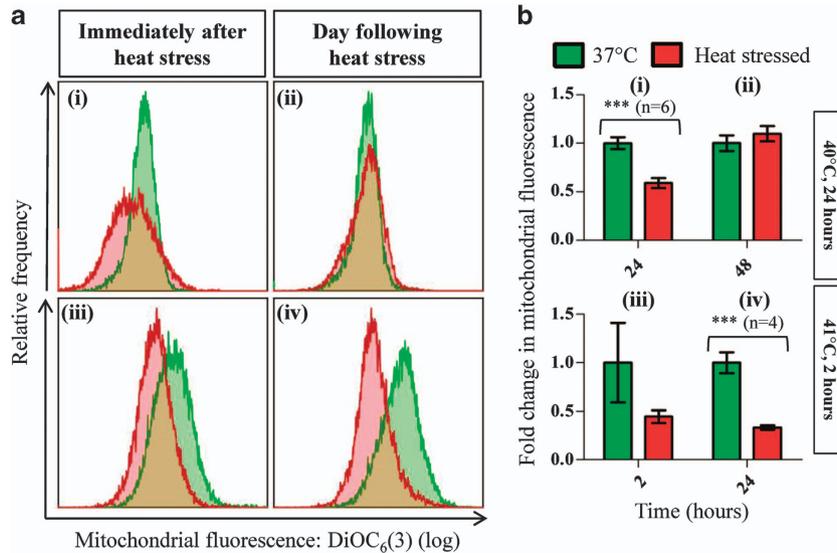
Figure 4 Heat stress induced mitochondrial depolarisation in early-stage parasites. Mitochondrial depolarisation was observed immediately following heat stress in early-stage parasites exposed to 40 °C for 24 h (**i**), although it was not noted the following day (**ii**). Early-stage parasites exposed to 41 °C for 2 h exhibited slight mitochondrial depolarisation immediately after heat stress (**iii**) that was significant the next day (**iv**). (**a**) Overlays of DiOC$_6$(3) fluorescence histograms show representative merged data sets of at least two replicates for each time point and condition as an individual peak. A decrease in the median fluorescence of a population is indicated by a shift to the left (depolarisation). Fluorescence values were taken as the median fluorescence of individual populations. (**b**) Bar graphs indicate the arithmetic mean of replicates ± S.E.M. Comparisons between control and heat-stressed parasites showed *P*-values that are significant at <0.001 (***). Heat stress conditions are indicated on the right
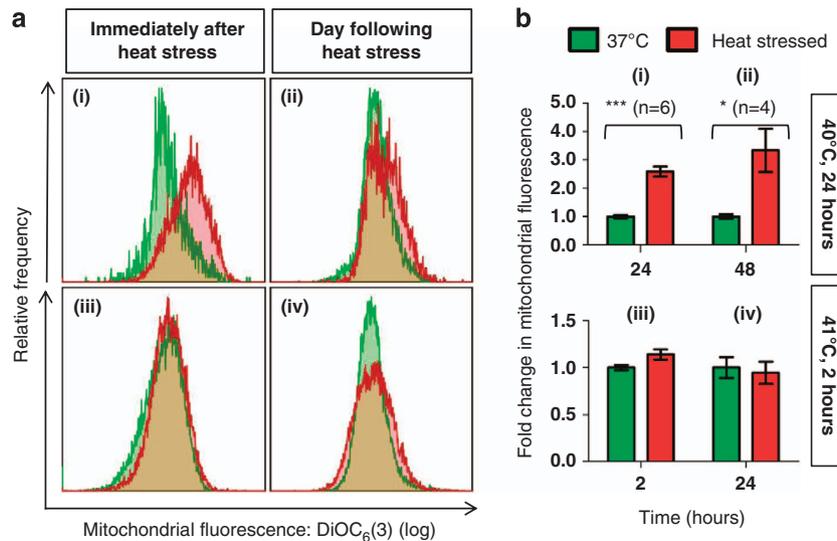

Figure 5 Heat stress-induced mitochondrial hyperpolarisation in late-stage parasites exposed to 40 °C for 24 h. Late-stage parasites exposed to 40 °C for 24 h showed mitochondrial hyperpolarisation after heat stress (**i**) and the following day (**ii**), whereas late-stage parasites exposed to 41 °C for 2 h showed practically no change in mitochondrial polarisation (**iii** and **iv**). (**a**) Overlays of DiOC$_6$(3) fluorescence histograms show representative merged data sets of at least two replicates for each time point and condition as an individual peak. An increase in the median fluorescence of a population is indicated by a shift to the right (hyperpolarisation). Fluorescence values were taken as the median fluorescence of individual populations. (**b**) Bar graphs indicate the arithmetic mean of replicates ± S.E.M. Comparisons between control and heat-stressed parasites showed *P*-values that are significant at <0.05 (*) or <0.001 (***). Heat stress conditions are indicated on the right

least partly explain the conflicting conclusions of previous studies.[6,7] More significantly, however, our data suggested that the PCD phenotype depends on the intra-erythrocytic developmental stage. Early-stage parasites showed biochemical markers of PCD that were reminiscent of an apoptosis-like form of PCD. The phenotype observed in late-stage parasites, which exhibited a different combination of biochemical markers as well as cytoplasmic vacuolisation and continued survival after heat stress despite no replication, appeared similar to autophagy-like cell death.

Give and take: the potential mutual benefits of fever in malaria. Central to this study was the following question: who benefits most from fever during malarial illness – parasite or
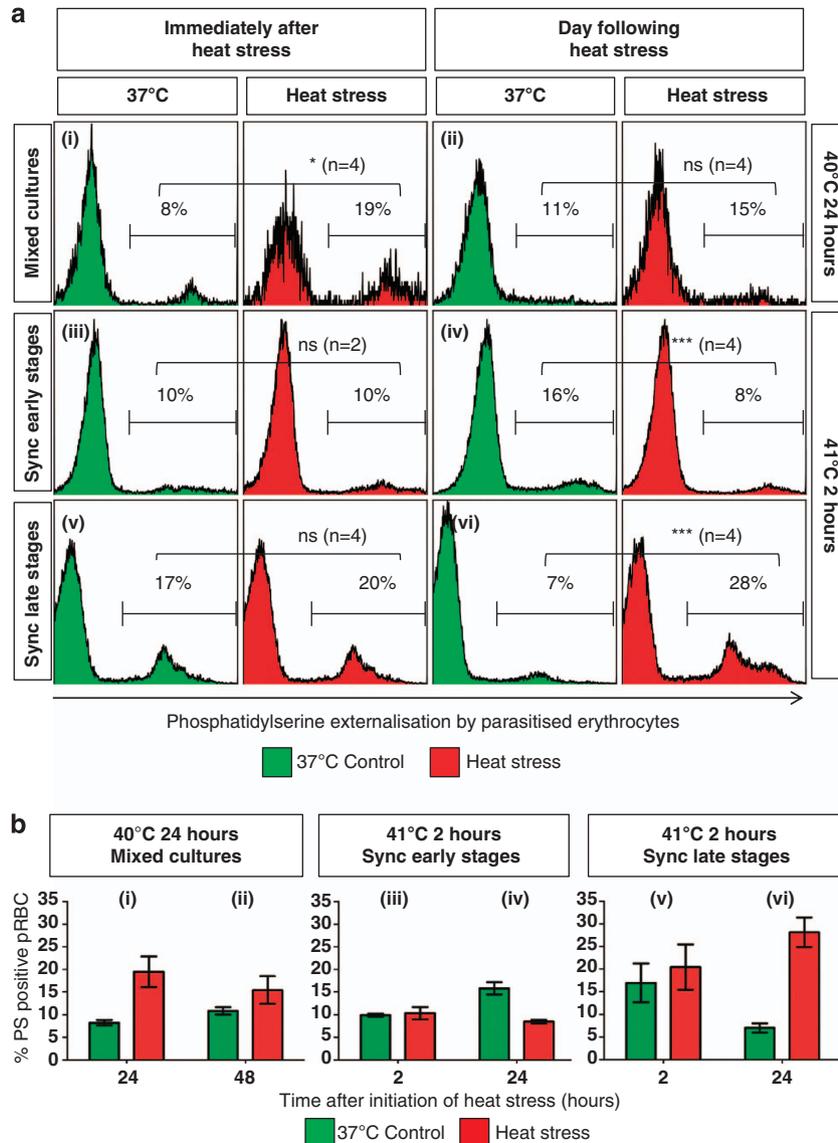
Figure 6 PS externalisation in pRBC exposed heat stress. (**a**) Fluorescence histograms and (**b**) bar graphs of pRBC showed an increase in PS externalisation by mixed-stage cultures immediately after exposure to 40 °C for 24 h (**i**) and was still apparent at 48 h (**ii**), although the latter difference was not statistically significant. No increase was noted in synchronised ring-stage pRBC immediately after exposure to 41 °C for 2 h (**iii**), with an apparent decrease noted at 48 h; however, it should be noted that the 37 °C control parasites used for comparison had developed to late stages, which typically have higher PS externalisation, even without stress. When comparing the heat-stressed culture at both time points (**iii** and **iv**), no change was apparent. Although PS externalisation was not seen in late-stage pRBC exposed to 41 °C for 2 h immediately after heat stress (**v**), an increase was observed a day later (**vi**). Representative histograms are merged data sets of at least two samples. Parasite stages are shown on the left as either mixed-stage cultures (Mixed) or synchronised (Sync) early- or late-stage cultures. Heat stress conditions are shown on the right. Gates on individual histograms indicate the percentage of infected erythrocytes that are PS positive. Comparisons between control and heat-stressed parasites have *P*-values that are significant at < 0.05 (*), < 0.001 (***) or not significant (ns)

host? On the one hand, fever may provide the host with a mechanism for parasite clearance.[5] Complete clearance would be most beneficial, but in this regard fever is relatively inefficient, as newly invaded parasites derived from the ruptured schizonts that caused the onset of fever are generally spared, even after high fever peaks.[23] It seems likely that parasites that survived exposure to 40 °C in this study were early-stage parasites. Our data showed that even after exposure to a high peak temperature of 41 °C, a significant number of ring-stage parasites and even some late-stage parasites survive, although a measurable increase

in parasitaemia was only noted after several days of continuous culture. On the other hand, fever may provide a number of benefits to the parasite, including accelerated parasite maturation, as well as increased cytoadherence and reduced deformability of pRBC, thought to aid in the sequestration of pRBC to microvasculature.[9–12] With every asexual parasite capable of producing as many as 32 new merozoites at the end of a 48 h life cycle,[24] uncontrolled parasite replication would place the host at risk of death before the slow-forming gametocytes could be transmitted. A potential mutual benefit of exposure to increased
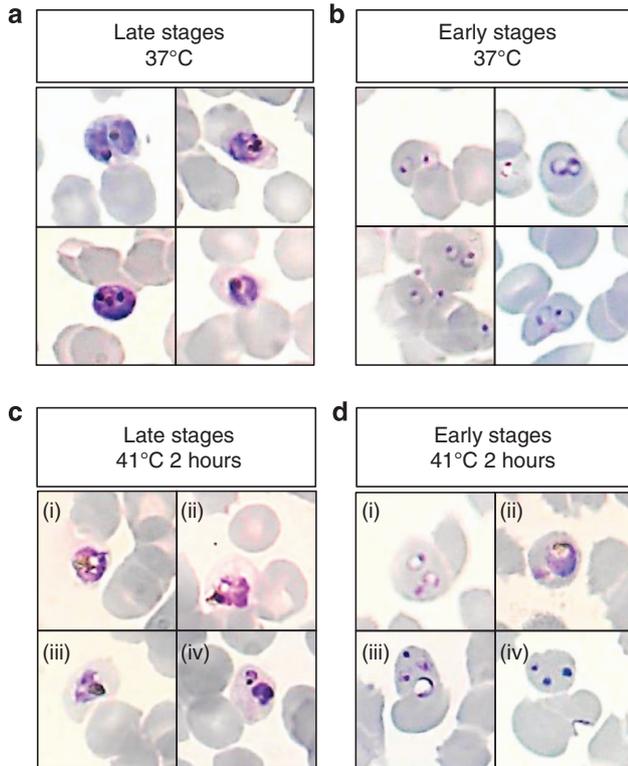
Figure 7 Heat stress induced abnormal morphology in *P. falciparum*. Control *P. falciparum* morphology showed trophozoites (**a**) that were large, full, well-rounded with clear and concentrated haemazoin and relatively even staining; and ring-stage parasites (**b**) that showed lightly-stained, delicate and rounded rings with single chromatin dots. In contrast, 24 h after exposure to 41 °C for 2 h, trophozoites (**c**) appeared unevenly stained with jagged borders and cytoplasmic vacuoles (**ci–iii**), or were condensed and very darkly stained (**civ**). Ring-stage parasites that were exposed to the same stress (**d**) showed variable morphology at 24 h. Some rings appeared slightly vacuolised (**di**) and remained as ring-stage parasites 24 h after exposure instead of progressing to trophozoites. Some morphologically normal trophozoites were also observed (**dii**). Many ring-stage parasites were abnormally large with a central vacuole (**diii**). Chromatin-like dots without any ring structure were also observed in many cells (**div**)

temperatures therefore lies in the induction of PCD: by providing the parasite with the means to self-limitation, the burden of infection on the host is reduced, although sufficient parasites are conserved for continued sublethal infection.[23,25] Our data suggest that intra-erythrocytic *P. falciparum* is likely to undergo PCD in response to heat stress.

PCD markers varied with life stages: different pieces, same puzzle? *P. falciparum* early and late developmental stages exhibited some intriguing differences in their response to the same stress conditions. Although ring-stage parasites exposed to 40 °C for 24 h exhibited the depolarisation that is expected to result from the permeabilisation of the outer mitochondrial membrane during apoptosis,[13] late-stage parasites exposed to the same stress surprisingly exhibited mitochondrial hyperpolarisation. Mitochondrial hyperpolarisation has been observed in protozoa other than *Plasmodium*. In heat-stressed *Leishmania*, hyperpolarisation formed an early part of an apoptosis-like cell death and was

correlated with increased mitochondrial respiration, which drove superoxide radical production, in turn causing oxidative damage that mediated cell death.[26] However, during apoptosis-like cell death in *Trypansoma*, mitochondrial hyperpolarisation was associated with decreased mitochondrial respiration.[27] Both mitochondrial depolarisation and hyperpolarisation may be apoptosis responses in trypanosomatids.[28] In metazoans, a transient increase in the $\Delta\Psi$m is thought to serve as a key checkpoint in determining cell fate,[27] and is correlated with increased production and accumulation of reactive oxygen species (ROS) by the mitochondria during apoptosis.[17,18] However, mitochondrial hyperpolarisation and increased ROS generation have also been implicated in the priming and occurrence of necrosis.[15,29,30] In *P. falciparum*, we observed continued mitochondrial hyperpolarisation of late-stage parasites after 24 h exposure to 40 °C and it is not entirely clear whether this marker is indicative of PCD or perhaps a necrosis-like form of cell death. Furthermore, the overall role of the mitochondrion in PCD in asexual *P. falciparum* may be questioned. Asexual stages of *P. falciparum* contain only a single, minimally active mitochondrion that functions primarily in *de novo* pyrimidine synthesis.[31]

We also observed differences between the PS externalisation of early- and late-stage pRBC exposed to 41 °C. In accordance with a previous study,[10] late-stage pRBC showed increased PS externalisation after exposure to heat stress. However, no change was observed in early-stage pRBC. During apoptosis, loss of plasma membrane asymmetry results in PS externalisation, which is often used as a marker of apoptosis and is thought to serve as a signal in mammalian cells for phagocytes to engulf and digest cellular remnants.[32] Would PS externalisation by pRBC be an advantage or disadvantage to the parasite residing within? Increased PS externalisation, observed both during parasite maturation[10,33,34] and after stress by febrile temperatures,[10] has been suggested to aid in the cytoadherence and sequestration of pRBC in microvasculature, thereby protecting parasites from splenic trapping and destruction.[34,35] In this study, PS externalisation was only observed in late-stage pRBC exposed to 41 °C a day after stress was withdrawn. PS externalisation also occurs during erythrocyte apoptosis,[36,37] and oxidative stress from *P. falciparum* infection has been shown to induce host cell apoptosis[38] and has been proposed to be the cause of PS externalisation in pRBC.[10] In this light, it is possible that increased PS externalisation by late-stage pRBC may simply be a by-product of oxidative stress from parasites.

Although biochemical markers of PCD are preferred over morphological criteria,[15] the assessment of autophagy is largely based on morphology.[39] Therefore, in light of the dissimilar responses of early- and late-stage parasites exposed to 41 °C, particularly the relative lack of biochemical markers of PCD exhibited by late-stage parasites despite a significant effect on parasite growth, morphological studies of both Giemsa-stained fixed slides and unstained, wet-mounted samples were also considered for parasites exposed to 41 °C.

Cytoplasmic vacuolisation was observed 1 day after heat stress for several subsequent days in late-stage parasites exposed to 41 °C for 2 h. Real-time microscopy showed that a

significant number of late-stage parasites exposed to 41 °C showed rapid movement within the food vacuole for several days after exposure to heat stress. Such movement is characteristic of healthy parasites[21] and has been shown to be decreased by the antimalarial drug artesunate, which also caused the appearance of 'pyknotic' parasites.[21,40] It has been suggested that loss of movement in the food vacuole is a very early indicator of an adverse parasite response to external stimuli that precedes other morphological changes.[40] Despite showing a significant number of parasites that were apparently still alive, parasites did not form new ring-stage parasites as would be expected of healthy parasites (Figure 2) and the appearance of new ring-stage parasites and increasing parasitaemia was delayed by several days (Supplementary Figure 1). The eventual recovery of parasitaemia and the formation of new ring-stage parasites indicate that at least a few synchronised late-stage parasites survived exposure to 41 °C. The appearance of cytoplasmic vacuolisation, along with the lack of growth exhibited by apparently living parasites, is suggestive of an autophagy-like form of PCD. Similar to Totino *et al.*,[41] who observed autophagy-like cell death in drug-treated *P. falciparum*, we also observed very little DNA fragmentation by the TUNEL assay, which was deemed not to be significant. 'Crisis form' morphology was also reported by Oakley *et al.*[7] under heat stress conditions similar to those employed by the present study.

Autophagy is a process of sequestration and subsequent degradation of cytoplasmic components that is regulated primarily by the sequential action of several autophagy-related (Atg) proteins. *Plasmodium* genomes encode a number of putative Atg orthologues,[42] although the most well-studied of these, the ubiquitin-like Atg8, has been shown to localise to the apicoplast, rather than the cytoplasm, in *P. falciparum*.[43] Therefore, it is unclear whether the *P. falciparum* proteome encompasses the tools required to undergo autophagy-like cell death, with very few observations of the phenomenon reported. However, it has been suggested that the sequestration of micronemes and rhoptries in double-membrane structures during the differentiation of sporozoites to trophozoites in hepatocytes may represent a type of functional autophagy in malaria parasites.[42] It is also not clear whether autophagy 'in dying cells is the cause of death or actually an attempt to prevent it',[39] particularly as autophagy often inhibits the induction of apoptosis or protects cells from exposure to apoptotic stimuli.[44] Our own observations reflect this conflicting nature: the slow decrease of living parasites observed by real-time microscopy suggests that parasites are undergoing cell death; however, the eventual recovery of parasitaemia suggests that at least some parasites survive this process. It has been suggested that apoptosis and autophagy-like cell death are forms of PCD that may share some common regulatory proteins and the two may be induced simultaneously.[44] We observed that the same stimulus that induced an apoptosis-like form of PCD in ring-stage parasites also induced autophagy-like cell death in late-stage parasites.

Apples and pears: the problem of identifying metazoan PCD phenotypes in *P. falciparum*. Despite mounting evidence suggesting that *P. falciparum* undergoes PCD in response to heat stress, the exact phenotype of cell death remains undetermined. Apoptosis and autophagy are recognised phenotypes of PCD, whereas necrosis is considered a non-PCD phenotype of cell death,[15] although some active form of necrosis might also be possible.[45] However, overlap between phenotypes may occur and many markers, including DNA fragmentation and mitochondrial depolarisation, may also occur during necrosis.[14] Our data provided evidence suggesting that the asexual blood stages of *P. falciparum* may be capable of undergoing both apoptosis-like and autophagy-like forms of PCD. However, *P. falciparum* may exhibit a unique PCD phenotype that could include such phenomena as both mitochondrial hyperpolarisation and depolarisation—observations that were attributed to apoptosis in other protozoans.[29] The origin of PCD may precede that of multicellularity[46] and thus with an organism as phylogenetically ancient and evolutionary distant from other taxa as *P. falciparum*,[47] attempting to impose metazoan PCD phenotypes may be misleading.

Conclusion

Intra-erythrocytic *P. falciparum* exhibited markers of an apoptosis-like PCD phenotype in ring-stage parasites and an autophagy-like cell death phenotype in late-stage parasites in response to heat stress. However, whether these are truly different phenotypes or simply different facets of a PCD phenotype unique to *P. falciparum* and distinct from metazoan apoptosis remains unclear. Elucidation of the underlying protein machinery responsible for the execution of PCD in *P. falciparum* will provide insight into the working of active cell death and the complex interactions between parasite and host.

Flow cytometry. Flow cytometric analyses were performed on a Beckman Coulter Gallios flow cytometer (Beckman Coulter Inc., Miami, FL, USA). Excitation for all assays was by 488 nm blue laser. Emission was detected with the use of 545/40BP (525 ± 20 nm, FL1) and, where indicated, 575/30BP (575 ± 15 nm, FL2) filters. Optical alignment was monitored daily with Beckman Coulter Flow Check Pro fluorospheres (Beckman Coulter Inc., Brea, CA, USA). Post-acquisition analyses were performed with Beckman Coulter Kaluza (v1.1) software.

TO flow cytometry for parasitaemia. Parasitaemia was measured daily by flow cytometry with the DNA-binding dye TO, similar to a previous method.[51] Whole-culture samples (10 μl) were diluted 100-fold to 1 ml in Sorenson's phosphate buffer (47 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7.2) with 1 μM TO final concentration (diluted from a 10 mM stock in methanol) and incubated at room temperature in the dark for 20 min. Stained cells were analysed by flow cytometry within 1 h. Erythrocytes were gated on a forward- *versus* side-scatter dot plot and analysed on a FL1 integral (log) histogram, with regions for uninfected, ring-infected and trophozoite- or schizont-infected erythrocytes delineated. Approximately 50 000 events in the erythrocyte gate were counted. Regions had previously been confirmed by microscopy of Giemsa-stained smears of synchronised cultures.

TUNEL assay for DNA fragmentation. The TUNEL assay was performed according to manufacturer's recommendations, with modifications similar to a previous study.[7] Briefly, *P. falciparum* cultures were centrifuged and the cell pellets fixed on ice for 60–90 min in 4% formaldehyde and phosphate-buffered saline (PBS: 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), followed by permeabilisation with 0.1% tri-sodium citrate (w/v) and 0.1% Triton X-100 (v/v) in PBS for 3 min on ice. Labelling with DNA-staining solution (including TdT enzyme and FITC-dUTP) was performed according to the manufacturer's recommendations for 60–90 min at 37 °C, followed by staining with propidium iodide (PI) for 30 min at room temperature. Labelled cells were analysed by flow cytometry within 3 h. PI-positive parasites were acquired on a FL2 time-of-flight (lin) *versus* FL2 integral (lin) dot plot, with gated parasites analysed on a FL1 integral (log) histogram for DNA fragmentation, measured as FITC-dUTP fluorescence. At least 10 000 PI-positive events were counted. DNase-treated, non-treated and unlabelled parasites were used as positive, negative and staining controls.

DiOC6(3) flow cytometry for $\Delta\Psi$m. *P. falciparum* cultures were diluted 20-fold in PBS, stained with 10 nM $DiOC_6(3)$ (diluted from a 100 mM stock in DMSO) and 50 μM HE (diluted from a 10 mM stock in DMSO) and incubated at 37 °C for 45 min in the dark. Following incubation, cells were washed and suspended in 1 ml PBS and analysed by flow cytometry immediately. Erythrocytes were gated on a forward- *versus* side-scatter dot blot. HE-positive pRBC counted on a FL2 integral (log) histogram were analysed for $DiOC_6(3)$ fluorescence on a FL1 integral (log) histogram. At least 50 000 events in the erythrocyte gate were counted. Positive controls were treated with 200 nM CCCP for 1 h before staining. Unstained cells, cells stained with only HE or $DiOC_6(3)$ and non-parasitised erythrocytes were used as staining controls. Mitochondrial depolarisation by the uncoupling agent carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, data not shown) confirmed the integrity of the assay.

Annexin V-FITC for PS externalisation. *P. falciparum* cultures were diluted 50-fold in PBS and stained sequentially with a final concentration of 50 μM HE (diluted from a 10 mM stock in DMSO) in PBS for 15 min at 37 °C in the dark and annexin V-FITC in 1X annexin-binding buffer (provided with the kit), according to the manufacturer's recommendations with modifications similar to a previous study,[10] for 15 min at room temperature in the dark. Stained cells were diluted to 1 ml in 1X annexin-binding buffer and analysed by flow cytometry within 1 h. Erythrocytes were gated on a forward- *versus* side-scatter dot blot and pRBC were discriminated on a FL 2 integral (log) histogram for HE fluorescence. Gated pRBC were analysed for annexin V-FITC fluorescence on a FL1 integral (log) histogram. At least 50 000 events in the erythrocyte gate were counted. Parasitised cultures treated with recombinant annexin V before staining were used as a negative control and unstained parasite cultures and parasite cultures stained with only HE or annexin V-FITC were used as staining controls.

Real-time microscopy. Samples of 41 °C heat-stressed and 37 °C control parasitised cultures were wet-mounted by placing 5 μl sample on a glass slide and covering the sample with a coverslip. Samples were observed with an Olympus

BX41 microscope under bright field and × 1000 magnification with an immersion oil type objective lens, at room temperature, with a halogen light source. Real-time microscopy was performed immediately after heat stress and for 5 subsequent days. Live images were captured with an Olympus XC50 digital camera. Parasites observed with rapid movement within the food vacuole were considered to be alive.[21]

Statistical analysis. Bar graphs were compiled using GraphPad Prism 5, with raw data values exported from analyses by Beckman Coulter Kaluza (v.1.1) software. Student's unpaired *t*-tests were performed with Microsoft Office Excel 2010 to test for significance between treated and control groups.

Conflict of Interest
The authors declare no conflict of interest.

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