Modulation of the expression of sirtuins and *var* genes by heat shock in the malaria parasite *Plasmodium falciparum*: a pattern emerges

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Abstract

Background: In the malaria parasite *Plasmodium falciparum* the expression of 'var' virulence genes is regulated through epigenetic mechanisms. Its sirtuin epigenetic regulators have a direct effect on var gene expression patterns, are increased in a laboratory strain of *P. falciparum* exposed to heat shock and are positively associated with fever. A Gambia study extended this association to blood lactate and var genes commonly expressed in severe malaria, and between *PfSir2A* and group B var. A Kenyan study extended this association to between *PfSir2A* and overall var transcript level. These observations suggest a mechanism through which stress phenotypes in the human host might be sensed via a parasite sirtuin, and virulence gene expression modulated accordingly.

Methods: *In vitro* experiments were conducted using laboratory and recently-laboratory-adapted Kenyan isolates of *P. falciparum* to follow up the correlative findings of the field study. To investigate a potential cause-and-effect relationship between host stress factors and parasite gene expression, qPCR was used to measure the expression of sirtuins and var genes after highly synchronous cultured parasites had been exposed to 2h or 6h of heat shock at 40°C or elevated lactate.

Results: Heat shock was shown to influence the expression of *PfSir2B* in the trophozoites, whereas exposure to lactate was not. After the ring stages were exposed to heat shock; sirtuins, severe-disease-associated upsA and upsB var genes and var genes in general were not altered. More biological replicate experiments will be needed to confirm our observations.

Conclusions: This study demonstrates that heat stress in laboratory and recently-laboratory-adapted isolates of *P. falciparum* results in a small increase in *PfSir2B* transcripts in the trophozoite stages only. By contrast, the association between hyperlactataemia and sirtuin/ var gene expression that was previously observed *in vivo* appears to be coincidental rather than causative.

Background

Several hundred thousand people die from malaria each year: in 2018, 405,000 deaths were recorded. This is almost always due to infection with the malaria parasite species *P. falciparum*, which can cause severe and lethal malaria, and children make up 67% of this mortality [1]. Severe malaria manifests as three major syndromes: cerebral malaria, acute respiratory distress due to metabolic acidosis and severe anaemia (mainly in young children) [2]. Severe malaria is defined by a group of clinical factors including fever of above 39°C [3, 4] and high blood lactate of ≥ 5 mM [1, 3, 5, 6]. Fevers usually last between 2 and 6 h following a malaria paroxysm [7], while hyperlactataemia can persist for variable periods [3].

Severe malaria has been associated, in multiple studies, with parasites expressing particular members of the var virulence gene family [8-14]. This is a large family of ~60 highly variable genes that all encode variants of the protein *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1). PfEMP1 is a key

virulence factor: it is exported to the surface of parasite-infected erythrocytes, where it mediates adhesion to host cell receptors found on the endothelial lining of blood vessels. Thus, infected erythrocytes can adhere in the microvasculature and avoid splenic clearance (reviewed in [15]). In addition, the host immune system can recognise these PfEMP1 adhesins [16], however, the parasite has evolved to switch their expression regularly [17, 18], allowing immune evasion [19] and thus persistent and repeated infections [20, 21].

Var genes are grouped into the groups upsA, B, C and E based on sequence similarities in their upstream region, as well as their chromosomal location and direction of transcription [22]. There appears to be a 'hierarchy' of switching, in which genes of the upsA group, encoding large and complex PfEMP1s that tend to correlate with severe disease [8-14], are expressed preferentially in immunologically naïve individuals [8]. There is some evidence that the upsB group also associates with severe malaria, whereas the upsC group usually does not [10, 11].

Var genes are generally considered to be expressed in a mutually exclusive fashion [18], although this may not be strictly true in all strains [23, 24]. Mutually exclusive expression means that at any one time, the majority of the family is silenced epigenetically via heterochromatin formation [25]. The *Plasmodium* sirtuins, PfSir2A and B, contribute to this regulation of *var* expression by enforcing histone deacetylation and thus heterochromatic silencing. Disruption of either sirtuin gene in cultured 3D7 parasites leads to deregulated expression of many *var* genes, with PfSir2A primarily controlling the groups upsA, C and E, while PfSir2B primarily controls the upsB group [26, 27].

These lines of evidence from *in vitro* cultured parasites informed a previous study of the correlation between sirtuin and *var* gene expression in *in vivo* human infections. qPCR was conducted on parasites directly isolated from Gambian patients with severe or mild malaria, revealing a correlation between elevated expression of *PfSir2A* and elevated expression of severe-disease-associated *var* genes. This further correlated with patient phenotypes of fever and hyperlactataemia, leading to the hypothesis that these indicators of host stress might be detected by the parasite and translated, via sirtuin activity, into altered patterns of *var* gene expression [11]. A separate study subsequently conducted on East-African (Kenyan) rather than West-African (Gambian) isolates reproduced the correlation between fever, high *PfSir2A* expression, low pre-existing anti-PfEMP1 immunity, and – in this study – a generally elevated level of *var* transcription rather than the elevation of any particular subgroup [28].

In this present study, conducted *in vitro* using recently-culture-adapted parasites, we endeavoured to establish whether the correlations detected in these field studies actually denote cause-and-effect relationships. Thus, indicating strategies evolved by the parasite to survive heat stress when a human host experiences malarial fevers. *P. falciparum* parasites were accordingly subjected to high temperature and high lactate levels *in vitro*, mimicking the high body temperature and hyperlactatemia that occur in severe malaria.

Methods

Parasite lines and culture

P. falciparum (3D7 (obtained from MR4) and 3 Kenyan strains (obtained from the European Malaria Reagent Repository, ID9775, ID3518 and ID10668)) were cultured in O⁺ erythrocytes obtained from the National Blood Transfusion Service (NBTS, Edgbaston, UK). Cultures were maintained at 4% haematocrit in Roswell Park Memorial Institute (RPMI) 1640 medium containing 25 µg/ml gentamicin sulphate, 0.2% sodium bicarbonate (Sigma, USA), 0.2% Albumax II (Life technologies, New Zealand), and 4.3% pooled human serum. Standard continuous culturing was carried out for 3D7 as described [29], or with more frequent media changes and at 1% haematocrit, as described [30] for the Kenyan strains. Staging and parasitaemia were assessed from a thin blood smear stained with Hemacolor® Rapid (VWR, UK). Storage and disposal of blood were in accordance with the Human Tissue Authority (HTA) license held by Keele University.

Synchronization of parasites

Double sorbitol synchronization was carried out as described [33], either 16 h apart (for the experiments with trophozoites) or 20 h apart (for the experiments with rings). A 20 h apart synchronization was required for the rings so as to ensure that synchrony is not lost as the parasites would have to invade new red blood cells and become rings again prior to the experiments.

Stress treatment of parasites

Stress treatment were carried out at 4% haematocrit. Trophozoites were subjected to high temperature (40°C) and/or to 5 mM sodium lactate for 2 or 6 h. Only two parasite strains, ID9775 and ID3518 were used for the experiments with the ring. They were exposed to these stressors at 2 ± 2 hours post invasion (hpi) for 6 h only. This level of heat shock was chosen because temperatures above 40°C were previously shown to kill most mature forms of the asexual stage [34] and in our laboratory [unpublished data]. Control samples, cultured under normal culture conditions, were included for all time points. After the stress, the medium was completely changed and the cultures were used for transcriptional analysis: RNA was extracted immediately, or after 10h recovery (in ring-stage experiments only). An aliquot of each culture was also diluted immediately after stress to parasitaemia of 0.5% (for trophozoites) or 0.1% (for rings) with 4% haematocrit in complete media, and this was then cultured for 48 h (for trophozoites) or 72 h (for rings) to determine survival rate. SYBR Green 1-based fluorescence assessment was conducted as described below. Survival was calculated using the equation: $(gt/gc) \times 100$, where gt = fluorescence of the test sample and gc = fluorescence of the control sample.

SYBR Green 1-based fluorescence assessment

Growth was assessed using the SYBR Green 1-based fluorescence method [35] by mixing 100 µl of the parasite cultures with 100 µl of lysis buffer containing SYBR green at a 1:5000 dilution (lysis buffer = 20mM Tris pH 7.5, 5mM EDTA, 0.008% v/v saponin and 0.08% v/v Triton X-100) in the wells of a 96-well black plate (CellStar, Greiner-bio-one, Germany) in the dark. The plates were then incubated for 1 h in the

dark and the fluorescence intensity was measured using 490 nm excitation and 510-570 nm emission filter in a Glomax-Multi Detection System (Promega, UK). Raw fluorescence values were used to determine statistical significance.

Gene expression analysis

Total RNA was extracted from each sample culture as described previously [37], but rather than adding Trizol to the iRBCs, Trizol was added directly to the parasite pellet after release from RBCs by saponin lysis at a concentration of 0.1%, (using equal volume of a 0.2% solution of saponin to the pelleted blood). Free parasites were briefly washing with ice-cold sterile 1xPBS. RNA yield was quantified using a Nanospec 1000 (Thermoscientific, USA). Contaminating DNA was removed using the wipe out buffer in the QuantiTect® Reverse Transcription Kit (QIAGEN GmbH, Germany). cDNA was synthesized with this kit and the absence of contaminating genomic DNA was verified by PCR across the intron of the gene PF3D7_0424300, as described previously [38].

Relative gene expression of the sirtuins and *var* genes was determined by qPCR in a StepOne Plus™ Real-time PCR machine (Thermo Fischer Scientific) using SensiFAST™ SYBR (Bioline, UK), cDNA (diluted at most 1:20) and primers (0.125 µM) in a 20 µl reaction volume. Cycling conditions were 50°C for 2 min, initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 54°C for 40 s and elongation at 60°C for 1 min. A melt-curve step was included to verify the specificity of the reactions. Reference genes used as controls were PF3D7_0717700 (serine tRNA-ligase), PF3D7_1444800 (FBP aldolase) and PF3D7_1246200 (actin). In ring-stage samples, the reference genes PF3D7_0501300 (SBP1) and PF3D7_1370300 (MAHRP1) were also measured as previously described [11] to confirm that parasites were consistently ring-stage. Data analysed with the inclusion of these reference genes are in Additional file 3. Primers to the target genes *PfSir2A*, *PfSir2B* [11], the major *var* gene groups [10], and the conserved acidic terminal segment (ATS) of all *var* genes [11] were used as published. Primers to *PfHsp70* (P1 and P2) and *MAHRP1* (P3 and P4) were designed using Snapgene software (Additional file 1, Table S1), their specificity was validated and minimal formation of primer-dimers was confirmed. All primers were manufactured and HPSF®-purified by Eurofins mwg®. Primer sequences are in Additional file 1 (Tables S2 and S3).

The relative copy number (RCN) of each target gene in each sample was calculated relative to the average of three housekeeping genes. RCN was calculated from $2^{-\Delta C_t}$. ΔC_t is the difference between the C_t of a target gene and the average C_t of three housekeeping genes, as shown in Additional file 2 alongside all raw qPCR data. Log10 of the mean RCN is represented in all graphs, and the RCN for each biological replicate experiment was used to determine statistical significance. Mean fold change in expression was calculated from the difference in the means of the control and stressed groups by determining the inverse of the antilog of this mean difference.

Statistical analysis

Experiments with the laboratory strain, 3D7, were done in independent biological triplicates (independent here means that a new culture of the same parasite strain was used to set up the same stress experiments on a different day), each measured by qPCR in technical triplicate (technical means that the same culture of a parasite line was analysed three times in different wells after a single stress experiment), thus yielding a total of 3 independent RCN values. Experiments with the three field strains were done in independent biological duplicates, also assessed by qPCR in technical triplicate, thus yielding 2 independent RCN values. Statistical analysis was conducted with Minitab 19 for Windows. A General Linear Model was performed with log₁₀ RCN as a response. Dichotomous categorical predictors of time, (2hrs or 6 hrs / 6hrs or 16hrs) temperature (37° or 40°C) and treatments (LAC 5mM or none). The log₁₀ RCN showed no evidence of heteroscedasticity using Levene's test. Anderson Darling detected some residuals were non-normally distributed, the GLM was assumed to be robust to this as n was at least 32 (ref). Multiple comparisons were done using Turkey's pairwise comparisons across the categorical predictors.

Results

Heat shock adversely affects parasite survival whereas lactate exposure does not

In order to choose appropriate parameters for exposing parasites to either heat shock or elevated lactate, we first measured parasite survival following such exposures, both at the ring stage and at the trophozoite stage. Trophozoites are known to be more sensitive to heat shock than rings [39], and although comparable stage-specific data are not published for lactate sensitivity, the trophozoite is also the most metabolically active stage, at which lactate production is highest. Cultures were treated at either 2 ± 2 hpi, or at 28 ± 4 hpi (primarily young rings and young trophozoites respectively). Exposure to stress was for 2 h or 6 h and consisted of heat shock at 40°C or 5 mM added lactate. These were chosen to mimic, respectively, common levels of fever in the human host and the WHO threshold for hyperlactataemia that defines severe malaria [2]. Survival was measured 48 h after treatment of the trophozoites or 72 h after treatment of the rings, thus allowing reinvaded rings to develop into trophozoites, when the DNA content can parallel parasite numbers. Four parasite strains were used: the reference strain 3D7 and three recently laboratory-adapted strains derived from Kenyan malaria patients.

In trophozoites, heat shock resulted in 0-20% death following a 2 h exposure and 20-40% death following a 6h exposure (Fig. 1A). The extent of heat-shock-induced death appeared strain-dependent, with one strain (ID9775) appearing more sensitive. This field strain (ID9775) grew most rapidly and robustly in normal culture conditions. By contrast to heat shock, lactate exposure did not appear to have an adverse effect on parasite survival (Fig.1A) and when both stressors were applied together, results were very similar to those seen after heat shock alone (Fig. 1A).

When heat shock was applied to ring-stage parasites, survival after 72h was more variable between strains than it had been at the trophozoite stage (Fig. 1 B). Kenyan strain ID3518 was almost completely insensitive, whereas the rings of strain ID9775 were affected (27% i.e. almost as severely affected as its

trophozoites). Lactate exposure for 6h at the ring stage again had little effect on parasite survival (Fig. 1 B).

Heat shock modulates the expression of PfSir2B in P. falciparum trophozoites

Having established heat shock conditions that caused only a moderate amount of parasite death in both trophozoites and rings, we measured the expression of sirtuin genes immediately after heat shock, as well as the expression of the gene encoding heat shock protein 70 (*hsp70*) as a positive control. These experiments were conducted first on trophozoites: the stage that was previously reported to upregulate *PfSir2A* expression after heat shock [34]. Across the strains, *hsp70* expression was indeed upregulated (Fig. 2A) by an average range of 3.42 – 4.92 folds (Table 1) in response to heat shock alone or with lactate but not to lactate exposure, as expected.

PfSir2A appeared to be unaffected by heat shock or lactate treatment (Fig. 2C, Table 1). *PfSir2B* expression was increased after 2 h of heat shock but not after 6 h (Fig. 2E) with mean fold changes ranging from 1.45 – 1.98 folds (Table 1). The lower end of the range involves heat shock in combination with lactate. This response was again heat-shock-specific because lactate versus no lactate was not significantly different, thus suggesting that heat shock was the main factor modulating the transcription of *PfSir2B*.

Table 1: Mean (95% CI) fold changes in the expression of *hsp70*, *Sir2A* and *Sir2B* in trophozoites after a 2 h or 6 h exposure to 5mM Lactate or 40°C heat shock, alone or in combination.

Gene/Time of exposure	Lactate 5mM	40°C	40°C + Lactate 5mM
<i>Hsp70</i> 2h	1.39 (0.49 to 3.89)	4.92 (1.73 to 14.06)	3.69 (1.06 to 12.88)
<i>Hsp70</i> 6h	1.06 (0.57 to 1.97)	4.70 (2.06 to 10.72)	3.42 (1.20 to 9.76)
<i>Sir2A</i> 2h	1.11 (0.43 to 2.87)	3.37 (0.57 to 3.26)	1.90 (0.64 to 5.64)
<i>Sir2A</i> 6h	1.06 (0.42 to 2.73)	3.21 (0.52 to 2.87)	1.23 (0.53 to 2.88)
<i>Sir2B</i> 2h	1.28 (0.59 to 2.76)	1.68 (1.11 to 3.20)	1.45 (0.76 to 2.77)
<i>Sir2B</i> 6h	1.07 (0.46 to 2.49)	1.98 (0.86 to 4.54)	1.48 (0.49 to 4.57)

Similarly, the expression of the sirtuins were measured immediately after the rings had been exposed to heat shock, elevated lactate, or both stressors combined for 6 h, i.e. expression was measured at 8 hpi. Sirtuins expression were also measured at 16 h (i.e. 10 h after the stressors were removed), at 18 hpi – around the time of maximum expression for active *var* gene(s) [41]. Only two strains (ID9775 and

ID3518) were selected for these experiments as mentioned previously. On exposure of ring-stage parasites to heat shock, *hsp70* was upregulated by an average of 1.98 (heat shock alone) or 1.64 folds (heat shock combined with lactate) after stress (Fig. 3B, Table 2). *Sir2A* (Fig. 3D) trended upwards both after stress and 10 h of recovery at 37°C (16 h after the onset of heat shock), while *Sir2B* (Fig. 3F) trended downwards only after stress, by ~ 2 folds (Table 2, (1/0.47 or 1/0.49 as it refers to a downregulation). These changes in *Sir2A* and *Sir2B* are not statistically significant. In addition, since the experiments involving the rings are less than 5 biological repeats with the 2 strains, the findings here can as best be described as preliminary.

Table 2: Mean (95% CI) fold changes in the expression of *hsp70*, *Sir2A* and *Sir2B* in rings after a 6 h exposure to 5mM Lactate or 40°C heat shock, alone or in combination, or after a 16 h recovery from ‘stress’.

Gene/Time of exposure	Lactate 5mM	40°C	40°C + Lactate 5mM
<i>Hsp70</i> 6h	0.62 (0.19 to 2.02)	1.26 (0.61 to 4.39)	1.98 (0.84 to 4.67)
<i>Hsp70</i> 16h	0.95 (0.57 to 1.58)	1.38 (0.78 to 2.46)	1.19 (0.69 to 2.05)
<i>Sir2A</i> 6h	1.02 (0.46 to 2.24)	1.28 (0.64 to 2.52)	1.28 (0.60 to 2.74)
<i>Sir2A</i> 16h	1.02 (0.56 to 1.87)	1.49 (0.73 to 3.05)	1.33 (0.64 to 2.76)
<i>Sir2B</i> 6h	0.98 (0.35 to 2.75)	0.47 (0.12 to 1.95)	0.49 (0.13 to 1.86)
<i>Sir2B</i> 16h	1.01 (0.64 to 1.58)	1.10 (0.72 to 1.69)	1.09 (0.63 to 1.87)

Alteration of var genes in P. falciparum ring stages after exposure to heat shock and lactate

The *var* gene expression were equally measured in the rings of the same previously strains immediately after exposure to heat shock, elevated lactate, or both stressors combined for 6 h, and at 16 h (i.e. 10 h after the stressors were removed) as previously described above. Since *var* gene families are hyper-diverse, *var* expression was measured using general primer sets that were previously developed to detect conserved regions within each ups-group of *var* genes [10]. These same primers were used in the study of Gambian field strains which originally reported the association between *var* gene expression, sirtuin expression, patient fever and hyperlactataemia [11].

Heat shock and lactate alone or in combination did not lead to a change in the expression of particular or specific *var* gene groups (Fig. 4A-I). Group A *var* genes that have been shown to be increasingly expressed in severe malaria were not selectively expressed. All *var* gene groups were expressed at similar levels in the control and treated/heat shocked parasites. Expectedly, the *var* genes

belonging to all the group except for group E (*var2csa* is the only *var* gene in the group) were upregulated after the 10 h of no stress at 18 hpi (p values are not shown for the time categorical factor) indicating that *var* genes are increasingly expressed with time.

Discussion

This is the first study to use recently laboratory-adapted field strains, rather than just the established laboratory parasite 3D7, to examine links between *P. falciparum* sirtuin expression and elevated temperature, *in vitro*. The data presented here suggests this link is more likely between *PfSir2B* and fever temperatures, however more data is needed to confirm this.

High body temperature and high blood lactate were previously shown to be associated with upregulated sirtuin and *var* expression in Gambian field isolates of *P. falciparum* causing severe disease [11]. In a separate study, fever alone was associated with increased expression of *PfSir2A* and *var* genes in Kenyan field isolates [28]. However, cause-and-effect relationships were not established in these studies. We have shown that *PfSir2B* can be increased in the trophozoites in response to heat shock, but this increase was not more than 2 folds on average. In addition, there was variability between the 3D7 biological replicates (Fig. 2A, first biological replicate showed a slight decrease). There is also a possibility that ring stages may respond to heat shock with a transient downregulation of *PfSir2B* and an upregulation of *PfSir2A*, but more biological replicate experiments are needed to establish this. It is possible that other factors that occur in *in vivo* during malaria may act in concert with heat shock to increase the expression of both sirtuins.

In general, the changes in *PfSir2B* expression seen in both previous and present studies were quite modest, and a significant change in the level of a transcript does not necessarily indicate a *biologically* relevant change. Possible changes in sirtuin expression may correlate with increased expression of *var* genes, as has been shown previously with the published findings from direct patient isolates [11, 28]. *Sir2A* and *var* genes have been shown to be increased in *in vitro* heat-shocked 3D7 parasites [34]. This was not seen in our study and although heat shock was applied for a longer time (6 h here vs 2 h), we may have missed the time when the 'stress' would have most likely led to an increased expression of *Sir2A* or *var* genes in the rings. However, since *var* genes appear to be increased with time [41], this may be unlikely. In addition, it is important to note that an asynchronous parasite population was used in this previously published study [34], thus making the observations in this laboratory study akin to that of the field studies where the ring stages of all ages can be found in circulation. In this study, we have tried to streamline expression profiles by using a highly synchronous parasite population.

In an attempt to validate the no change in *Sir2A* expression after heat shock, we designed a second independent approach to measure this response (<https://dx.doi.org/10.17504/protocols.io.btsxnnfn>). A luciferase reporter gene was cloned under the presumptive *Sir2A* promoter (~1.7kb of the gene's upstream sequence). The resulting plasmid, pLNSir2aproluc (Additional file 3, Figure S1A), was transfected into the 3D7 strain and the reporter gene was shown to follow an expression profile similar to that of the

endogenous *Sir2a* gene across the intraerythrocytic cycle [40](Additional file 3, Figure S1B).

Unfortunately, this system proved unsuitable for heat shock experiments because the luciferase was severely destabilised at 40°C: activity dropped by ~80% after a 2 h heat shock, irrespective of the promoter driving luciferase expression (Additional file 3, Figure S1C), thus obscuring any promoter-mediated regulation at the transcriptional level.

Finally, during the preparation of this manuscript, a third study was published examining *var* and *PfSir2A* expression in parasite-positive individuals in Mozambique [42]. Unlike the Gambian and Kenyan patients, these subjects were identified via a community survey, with none being severely ill or attending a clinic, although some did have an actual or reportedly-recent fever. *PfSir2A* transcription was actually lower in the febrile group than in the afebrile group – perhaps suggesting that our hypothesis concerning *PfSir2A* levels may only be significant in cases of more severe and symptomatic disease.

The no change in the expression of *var* genes and *Sir2A* and the very little change in the expression of *Sir2B* in the parasites in response to heat shock suggests that the host's immune system among other host factors occurring *in vivo* in severe vs uncomplicated malaria may be involved in the alteration of these genes as per observations [11, 28]. Antibodies have been shown to select against the global expression of *var* genes [28] and switching of *var* genes do occur as the parasite tries to conceal itself from the host's antibodies [17, 18]. In addition, specific *var* gene groups may not be impacted as the expression of a particular *var* gene by a parasite population is not ideal. Thus, a study attempting to isolate the effect of specific host factors in a parasite population outside the host will be problematic.

By contrast with the results discussed above concerning the relationship between fever and virulence gene expression, we did not detect any comparable response to elevated lactate levels. This suggests that although hyperlactataemia was clearly identified in our original field study as a correlate of sirtuin upregulation [11], the correlation is probably not causative. Alternatively, the level of lactate used here may not have been high enough to see an effect – 5mM is at, not above, the clinical threshold. However, only 26% of the original patient cohort in the Gambian study [11] had lactate levels >5mM, suggesting that *in vivo* this is indeed relatively rare, and aberrantly high.

Conclusion

Overall, the data presented here suggest that observations made in the West-African field study of patients with severe versus mild malaria [11] do reflect a causative relationship between fever in the human host and expression of *PfSir2B* in the trophozoite stages, but not between fever and the sirtuins or *var* virulence genes in the ring stages. The modest increase of *PfSir2B* only in the trophozoites and no change in sirtuin or *var* genes in the rings indicates a challenge in recapitulating *in vivo* observations using an *in vitro* approach. Furthermore, the ability of sirtuin activity to bring about a change in *var* transcription directly can be investigated with experiments, such as *var* gene ChIP for modified histones, or generation of sirtuin knockouts, which would be challenging in newly-cultured field strains. Also, the interplay between *PfSir2A* and *PfSir2B* remains uncharacterised and is probably complex.

List Of Abbreviations

DNA - Deoxyribonucleic acid

EDTA - Ethylenediaminetetraacetic acid Hsp70 – Heat shock protein-70

MR4 - The Malaria Research and Reference Reagent Resource Centre

MSF – Malaria SYBR Green 1-based fluorescence assay

PfEMP1 - *P. falciparum* Erythrocyte Membrane Protein 1

PBS - Phosphate-buffered saline

PfSir2A – *Plasmodium falciparum* silent information regulator 2A

PfSir2B - *Plasmodium falciparum* silent information regulator 2B

RBC – Red blood cell

RNA - Ribonucleic acid

qPCR - quantitative polymerase chain reaction

WHO – World health organization

Declarations

Ethics approval and consent to participate

Not applicable. All parasite strains were obtained from registered repositories, rather than directly from human patients. De-identified human erythrocytes in which *P. falciparum* strains were cultured was obtained from the National Blood Service. The use of blood is regulated under the Human Tissues Act 2004 and Keele University is an approved user of the National Blood Service.

Consent for publication

Not applicable

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LOA conducted experiments, designed some aspects of the study, analysed data with the assistance of DRH and drafted the manuscript. PDH assisted in the design of the luciferase experiments, provided the control luciferase-expressing strain and reviewed the manuscript. SJC supervised the study. All authors read and approved the final manuscript.

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Figures

Figure 1

Post-stress survival of *P. falciparum* after exposure to lactate and heat shock. A. Parasite survival 48 h after stress treatment for 2 h or 6 h at the trophozoite stage, relative to growth of a control culture, measured via DNA content in a SYBR-Green-1 based assay. B. Parasite survival 72 h after stress treatment for 6 h at the ring stage, relative to growth of a control culture, measured via DNA content in a SYBR-Green-1 based assay. Blue bars, 3D7; red bars, ID3518; green bars, ID9775; purple bars, ID10668.

Mean survival rates are from biological duplicates for the Kenyan strains, or triplicates for 3D7. Error bars represent standard error.

Figure 2

Altered expression of Hsp70 and PfSir2B in response to heat shock in *P. falciparum*. Log10 mean relative copy number of Hsp70 in trophozoites (A) or rings (B), Sir2A in trophozoites (C) or rings (D) and Sir2B in trophozoites (E) or rings (F) stages of parasites after stress treatments. Grey, purple, green and orange are data from strains 3D7, ID3518, ID9775 and ID10668, respectively. Circles, inverted triangles and squares are data from the first, second and third biological replicate experiments respectively. Error bars represent standard error mean. For Hsp70 and Sir2B, respectively, $p = 0.000$ (trophozoites) / 0.004 (rings) and 0.027 for the difference in the comparison of the temperature categorical factor (40°C (with or without lactate) vs 37°C (with or without lactate)).

Figure 3

Var gene transcription in ring-staged *P. falciparum* parasites after stress. Log₁₀ mean relative copy number of GrpA1 (A), GrpA2 (B), GrpA3 (C), GrpB1 (D), GrpB2 (E), GrpC1 (F), GrpC2 (G), GrpE (H) var genes and their ATS (I) in parasites after 6 h of stress treatments and after a further 10 h of no stress ('recovery', at 16 h from the time the stress was applied). Purple and green are data from strains ID3518 and ID9775, respectively. Circles and inverted triangles are data from the first and second biological replicate experiments respectively. Error bars represent standard error mean.

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