Heat Shock Modulates The Expression of Sirtuins and *Var* Genes in The Malaria Parasite *Plasmodium Falciparum*

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Research

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Abstract

Background: In the malaria parasite *Plasmodium falciparum* the expression of '*var*' virulence genes is regulated through epigenetic mechanisms. Two deacetylase enzymes of the sirtuin family have been implicated in this epigenetic control in laboratory-adapted parasites. A previous study of *var* gene expression in parasites isolated directly from Gambian malaria patients found that high expression levels of severe-disease-associated *var* variants correlated with high expression of the *PfSir2A* sirtuin, and these expression patterns also correlated with patient phenotypes of fever and hyperlactataemia. Together, the 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 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 cultured parasites had been exposed to 2h or 6h of heat shock at 40°C or elevated lactate at 5mM.

Results: Heat shock was shown to influence the expression of both sirtuins and *var* genes, whereas exposure to lactate was not. Heat shock in the trophozoite stage resulted in modest upregulation of the expression of sirtuins, particularly *PfSir2B*, by 2-3 fold in all strains tested. Interestingly, when heat shock was applied in ring stages *PfSir2A* was still upregulated but *PfSir2B* was downregulated. This correlated with a general upregulation of ring-stage *var* transcription, and particularly of severe-disease-associated upsA and upsB *var* genes, but there was no clear pattern in the dominant *var* gene(s) ultimately expressed by heat-shocked parasites.

Conclusions: This study demonstrates for the first time that heat stress in recently-laboratory-adapted isolates of *P. falciparum* results in altered sirtuin expression – *PfSir2B* as well as *PfSir2A* – and also the upregulation of *var* gene expression. These may be strategies evolved by the parasite to survive heat stress when a human host experiences malarial fevers. 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]). However, since the host immune system can recognise these PfEMP1 adhesins [16], 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 subgroups 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 indictors 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. *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

The reference strain of *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 for the Kenyan strains with more frequent media changes and at 1% haematocrit, as described [30]. 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.

Cloning and expression of luciferase reporter gene

The firefly luciferase gene (*Fluc*) was amplified from an existing expression cassette [31] using primers P1 and P2 (Additional file 1, Table S1), and ligated into the pLNSir2GFP plasmid [11] between the *Afl*II and *Avr*II sites. Cloning was conducted in the PMC103 strain of *E. coli* (ATTC). The resulting plasmid, pLNSir2aproluc (Additional file 2, Figure S1A), was transfected into the 3D7 strain as previously described [32].

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).

Stress treatment of parasites

Trophozoites were subjected to high temperature (40 °C) and/or to 5 mM sodium lactate for 2 or 6 h. The rings 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 shown in our preliminary work and also in previously published work [34] to kill most mature forms of the asexual stage. 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 10 h 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. For this, parasite counting by microscopy was conducted against up to 3000 erythrocytes and SYBR Green 1-based fluorescence assessment was conducted as described below. Survival was calculated using the equation: (gt/gc) x 100, where gt = parasitaemia or fluorescence of the test sample and gc = parasitaemia or 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 = 20 mM Tris pH 7.5, 5 mM 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.

Luciferase reporter assay

Stress experiments were conducted in the 3D7 *PfSir2A-luc* reporter line as above, using trophozoite stages only. A previously characterised luciferase reporter line, Dd2 *Pfpcna-luc* [31] was also included as a control in these experiments. Bioluminescence of the transgenic lines was measured immediately after stress using the improved Single-Step Lysis Protocol [36] in a Glomax-Multi Detection System (Promega, UK). Measurements were converted to relative luciferase activity as compared to the untreated transgenic line.

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 and brief washing with ice-cold PBS. 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* (P3 and P4) and *MAHRP1* (P5 and P6) were designed using Snapgene software, 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. All raw qPCR data are in Additional file 4.

The relative copy number (RCN) of each target gene in each sample was calculated relative to the average of three housekeeping genes. The relative quantity (RQ) of each target gene in each stressed sample was then further calculated as a proportion of that gene in the unstressed control sample. RQ was calculated by standard $2^{-\Delta\Delta Ct}$ analysis, deriving ΔCt from the difference between the Ct of a target gene and the average Ct of three housekeeping genes; ΔΔCt from the difference between the ΔCt in the stressed sample and the ΔCt in the control; and RQ from $2^{-\Delta\Delta Ct}$. RCNs were used to determine statistical significance and mean RQs are represented in all graphs.

Statistical analysis

Experiments with the laboratory strain, 3D7, were done in independent biological triplicates, each measured by qPCR in technical triplicate, thus yielding a total of 9 RQ values. Experiments with the three field strains were done in independent duplicates, also assessed by qPCR in technical triplicate, thus yielding 6 RQ values. Statistical analysis was conducted with GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA). Raw data were tested for normality, using the Kolmogorov-Smirnov test for normality, and the majority of the datasets did not follow a normal distribution. Statistical significance was therefore determined by using the Kruskal-Wallis test on all groups of RCN values (n = 27), followed by Dunn's post-test. A Kruskal-Wallis test p value of less than 0.05 indicated that one or more of the treatments caused statistically significant changes. The Dunn's post-test then determined which of the groups was statistically different.

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 parasitaemia can be easily assessed. 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 6 h exposure (Fig. 1A). The extent of heat-shock-induced death was strain-dependent, with

one Kenyan strain (ID9775) being markedly more sensitive than the other two, and 3D7 being of intermediate sensitivity. Interestingly, we observed that the most heat-sensitive field strain (ID9775) was the one that grew most rapidly and robustly in normal culture conditions. These results, measured via the SYBR Green-1 Fluorescence method, which measures levels of parasite DNA, were broadly corroborated by microscopy, conducted on two of the four strains as an independent measure of viable parasite numbers (Fig. 1B). Microscopy tended to yield a lower survival rate, because the SYBR Green-1 Fluorescence method, as used here, also measures background fluorescence from potentially 'dead' parasite DNA, whereas microscopy allows live and dead parasites to be distinguished. By contrast to heat shock, lactate exposure did not have a significant adverse effect on parasite survival (Fig. 1A, B) and when both stressors were applied together, results were very similar to those seen after heat shock alone (Fig. 1A, B).

When heat shock was applied to ring-stage parasites, survival after 72 h was more variable between strains than it had been at the trophozoite stage (Fig. 1C, D). Kenyan strain ID3518 was almost completely insensitive, whereas rings of strains ID9775 and ID10668 were significantly affected (27% and 38% death, respectively, i.e. almost as severely affected as their trophozoites). As before, microscopy yielded a more severe assessment of heat-induced death than the SYBR Green-1 Fluorescence assessment (Fig. 1C cf Fig. 1D). Lactate exposure for 6 h at the ring stage again had little effect on parasite survival (Fig. 1C, D).

Heat shock modulates the expression of sirtuins 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]. *Hsp70* expression was indeed upregulated, 3–7 fold in most strains, with considerable strain-to-strain variation ($p < 0.0001$) (Fig. 2A). This response was heat-shock-specific because *hsp70* was not upregulated after lactate exposure, as expected.

PfSir2A expression trended upwards in most strains after heat shock, particularly at the 2 h time point and somewhat less so at the 6 h time point. However, upregulation was usually less than 2-fold and did not reach statistical significance (Fig. 2B). *PfSir2B* expression, meanwhile, also trended upwards, generally by 2-3-fold, and reached statistical significance in several strains ($p = 0.0031$ at 2 h; $p = 0.0495$ at 6 h) (Fig. 2C). There was no significant difference in the response to heat shock alone versus heat shock combined with 5 mM lactate, thus suggesting that heat shock was the main factor modulating transcription of the genes measured here.

Since the *Sir2A* gene appeared only modestly upregulated after heat shock, we designed a second independent approach to measure this response. A luciferase reporter gene was cloned under the presumptive *Sir2A* promoter (~ 1.7 kb of the gene's upstream sequence) and the reporter gene was

transfected into 3D7 parasites, where it was shown to follow an expression profile similar to that of the endogenous *Sir2a* gene across the intraerythrocytic cycle [40](Additional file 2, Figure S1B). Unfortunately, although this system may be useful in other applications, it 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 2, Figure S1C), thus obscuring any promoter-mediated regulation at the transcriptional level.

Heat shock modulates the expression of sirtuins and var genes in P. falciparum ring stages, whereas lactate does not

Figure 2 shows that exposure to heat shock can modulate the expression of both the parasite sirtuins, particularly *PfSir2B*, at least in trophozoite-stage parasites. We therefore proceeded to investigate the same responses in ring-stage parasites, i.e. the stage at which *var* genes are also expressed. Sirtuin and *var* gene expression 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. The same genes were also measured 10 h later, at 18 hpi – around the time of maximum expression for active *var* gene(s) [41]. Kenyan field strains (ID9775 and ID3518) were selected for these experiments and, 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].

Exposure of ring-stage parasites to heat shock induced the upregulation of *hsp70* (Fig. 3A), as was previously seen in trophozoites (Fig. 2A). Upregulation was at most 2.5-fold and the magnitude of the response varied between strains, with strain ID9775 appearing almost entirely refractory at the ring stage, despite being clearly responsive at the trophozoite stage. *PfSir2A* expression trended upwards in both strains immediately after heat shock, similar to the response in trophozoites, but the increase was again less than 2-fold and did not reach statistical significance. Nevertheless, after 10 h of recovery at 37 °C, *PfSir2A* expression remained elevated, reaching statistical significance in strain ID3518 (Fig. 3B). *PfSir2B*, meanwhile, showed a response that contrasted with its response in trophozoites: immediately after heat shock, *PfSir2B* expression was downregulated by ~ 2-fold, but returned to baseline levels following 10 h of recovery (Fig. 3C).

In concert with these changes in sirtuin expression, changes in *var* gene expression after heat shock were also measured (Fig. 3D, E). Immediately after heat shock (i.e. 8 hpi), both strains showed a general upregulation of *var* transcription: 1.4-1.6-fold upregulation was measured with the pan-*var* 'ATS' primer (which detects the conserved sequence encoding the 'Acidic Terminal Segment' of PfEMP1), although this did not reach statistical significance. Primers specific to the various ups groups A-E suggested that there

was particularly significant upregulation of subtelomeric upsA and upsB genes in one of the two strains, ID3518 (Fig. 3D). Interestingly, 10 h later at 18 hpi the overall level of *var* transcript remained elevated, by ~ 5-fold in the more strongly responding ID3518 strain and ~ 1.6-fold in strain ID9775. However, the most strongly upregulated *var* gene had changed in both strains. A upsC gene was elevated in strain ID3518, while strain ID9775 had upregulated the upsE gene *var2csa* (Fig. 3E).

Having established that heat stress could indeed induce changes in both sirtuin and *var* gene expression when applied to ring-stage parasites, we proceeded to compare this with the transcriptional response to lactate exposure. This caused no consistent change in sirtuin expression (Fig. 4A, B), as had previously been shown in trophozoites, and there was also no clear change in *var* gene expression at the peak 18 hpi time point. When *var* expression was measured in young rings at 8 hpi, immediately after lactate exposure, one of the two strains did show a marked upregulation in groups B and C2 *var* transcripts (Fig. 4C). This, however, was not sustained by 18 hpi (Fig. 4D), casting some doubt upon its potential phenotypic significance.

Finally, we tested the transcriptional effect upon ring-stage parasites of combining heat shock with lactate exposure. In trophozoites, this had yielded similar results – in terms of both parasite survival and *hsp70*/sirtuin expression – as had heat shock alone. The same was evidently true in ring-stage parasites: *hsp70*, *PfSir2A* and *PfSir2B* expression all responded almost identically regardless of the presence or absence of 5 mM lactate (compare Fig. 5A-C with 3A-C). *Var* expression responses were likewise broadly similar (Fig. 5D, E) i.e. there was an overall upregulation in transcript levels, stronger in strain ID3518 than ID9775.

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. It is also the first to report a link between *PfSir2B*, as well as *PfSir2A*, and fever temperatures.

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. Here we have shown that trophozoites of *P. falciparum* consistently increased their expression of both sirtuins after heat shock, with *PfSir2B* responding more strongly than *PfSir2A*, whereas ring stages responded to heat shock with a transient downregulation of *PfSir2B* and an upregulation of *PfSir2A.* Our results are partially consistent with a previous *in vitro* study of the heat shock response in 3D7 [34], which found *PfSir2A* to be upregulated by 2.4-fold after a 41 °C heat shock. *PfSir2B*, however, did not reach a 2-fold threshold in this previous study, probably because asynchronous cultures were used rather than the synchronous cultures used in this study, thus masking the stage-specific changes that we observed here in *PfSir2B.*

In general, the changes in sirtuin expression seen in both previous and present studies were quite modest, in the 2-fold range, and a statistically significant change in the level of a transcript does not necessarily indicate a *biologically* significant change. However, these changes in sirtuin expression correlated with increased expression of *var* genes, consistent with the published findings from direct patient isolates [11, 28] and from *in vitro* heat-shocked 3D7 parasites [34]. In fact, the levels of *var* gene upregulation were higher in this study than reported previously [34], possibly because heat shock was applied for longer (6 h vs 2 h) or because our field strains were more responsive than the 3D7 laboratory strain. Furthermore, there was predominant expression of severe-disease-associated *var* genes in early rings immediately after heat shock: a novel finding in this study, and one that might reflect heat-induced changes to the structure of subtelomeric chromatin where upsA and upsB *var* genes are encoded. This, however, was not sustained at 18 hpi, by which time overall transcript levels remained elevated but a different *var* gene was predominantly upregulated in both strains.

There is conflicting literature on the subject of whether elevated *var* expression actually leads to elevated expression of PfEMP1 on the surface of the infected RBC [34, 42] and this question was not re-addressed in our present study. However, if it does then this could be a mechanism enabling parasites to cytoadhere more efficiently and thus survive better following an immune-response-induced fever. A second theory has also been published, in which *P. falciparum* immune evasion during chronic infections involves low *var* gene expression [28]. It may be that this indeed occurs in semi-immune individuals, who are less likely to experience symptomatic fevers, whereas parasites in symptomatic, minimally-immune individuals may tend to respond differently, by increasing *var* expression and cytoadherence. Finally, during the preparation of this manuscript, a third study was published examining *var* and *PfSir2A* expression in parasite-positive individuals in Mozambique [43]. 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 is only significant in cases of more severe and symptomatic disease.

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 – 5 mM is at, not above, the clinical threshold. However, only 26% of the original patient cohort in the Gambian study [11] had lactate levels > 5 mM, suggesting that *in vivo* this is indeed relatively rare, and aberrantly high.

Conclusion

Overall, the data presented here suggest that observations made in both East- and West-African field studies of patients with severe versus mild malaria [11, 28] do reflect a causative relationship between fever in the human host and expression of *var* virulence genes. Our study does not actually prove that the

change is mediated via sirtuin activity, but it does correlate with changes in sirtuin transcription (further proof would require experiments, such as *var* gene ChIP for modified histones, or generation of sirtuin knockouts, which would be challenging in newly-cultured field strains). Nevertheless, the idea is consistent with some existing data from the 3D7 strain showing that *PfSir2A* overexpression can correlate with *var* gene upregulation [11]. The interplay between *PfSir2A* and *PfSir2B* remains uncharacterised and is probably complex. However, the upregulation of Sir2A versus down-regulation of Sir2B that we observed when ring stages experienced heat shock may point to opposing roles for these two enzymes. This is a potentially interesting area for future study.

List Of Abbreviations

DNA - Deoxyribonucleic acid

EDTA - Ethylenediaminetetraacetic acid Hsp70 – Heat shock protein-70

MR4 - The Malaria Research and Reference Reagent Resource Center

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. CJM designed the study, supervised the study with the assistance of SJC, and wrote the manuscript. All authors read and approved the final manuscript.

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Figures

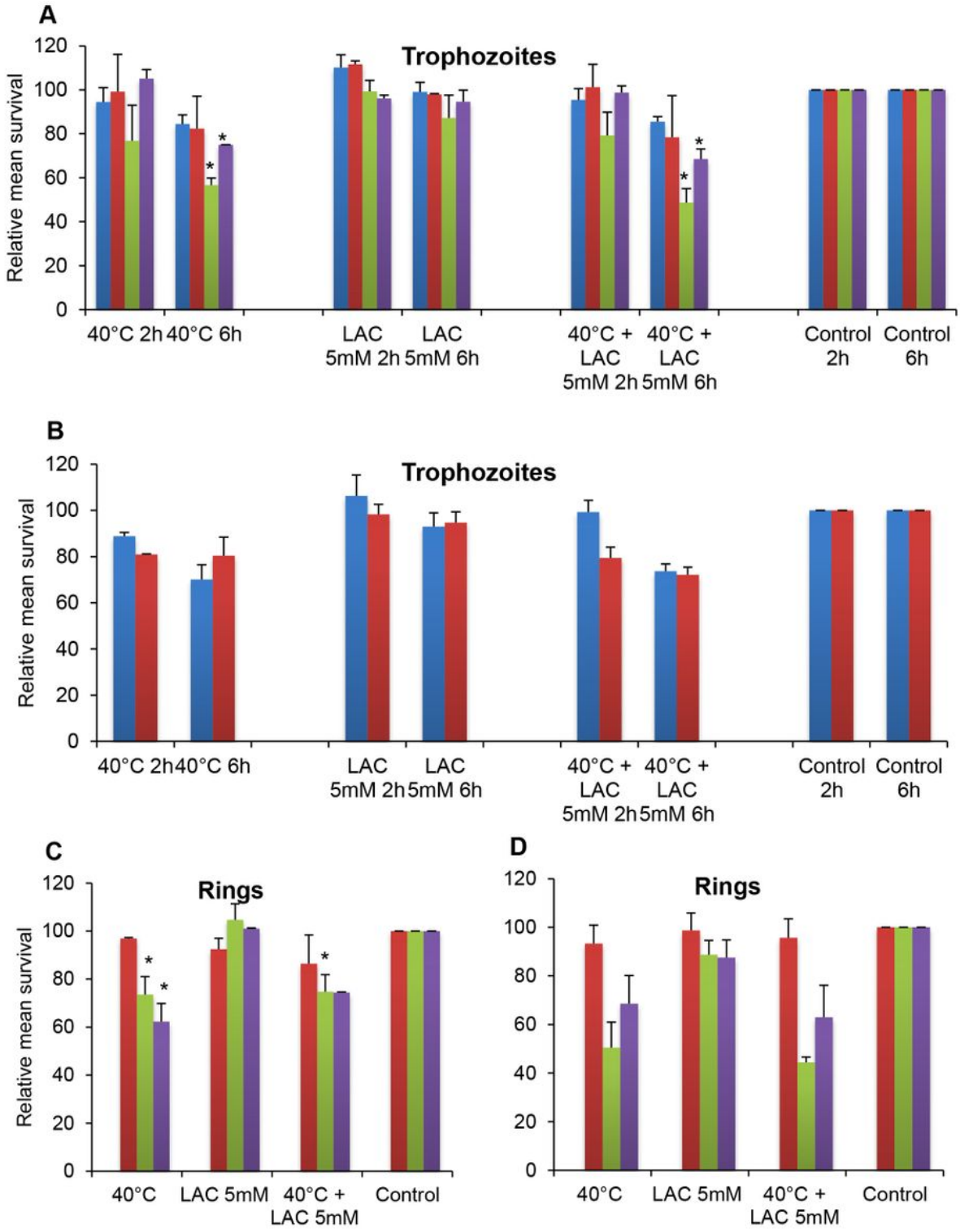

Figure 1

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 as in A, assessed by microscopy. C. 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. D. Parasite survival as in C.,

assessed by microscopy. Blue bars, 3D7; red bars, ID3518; green bars, ID9775; purple bars, ID10668. Mean survival rates are from biological duplicates for the African strains, or triplicates for 3D7. Error bars represent standard error. *, $p < 0.05$, Kruskal-Wallis test with Dunn's post test on raw fluorescence reads.

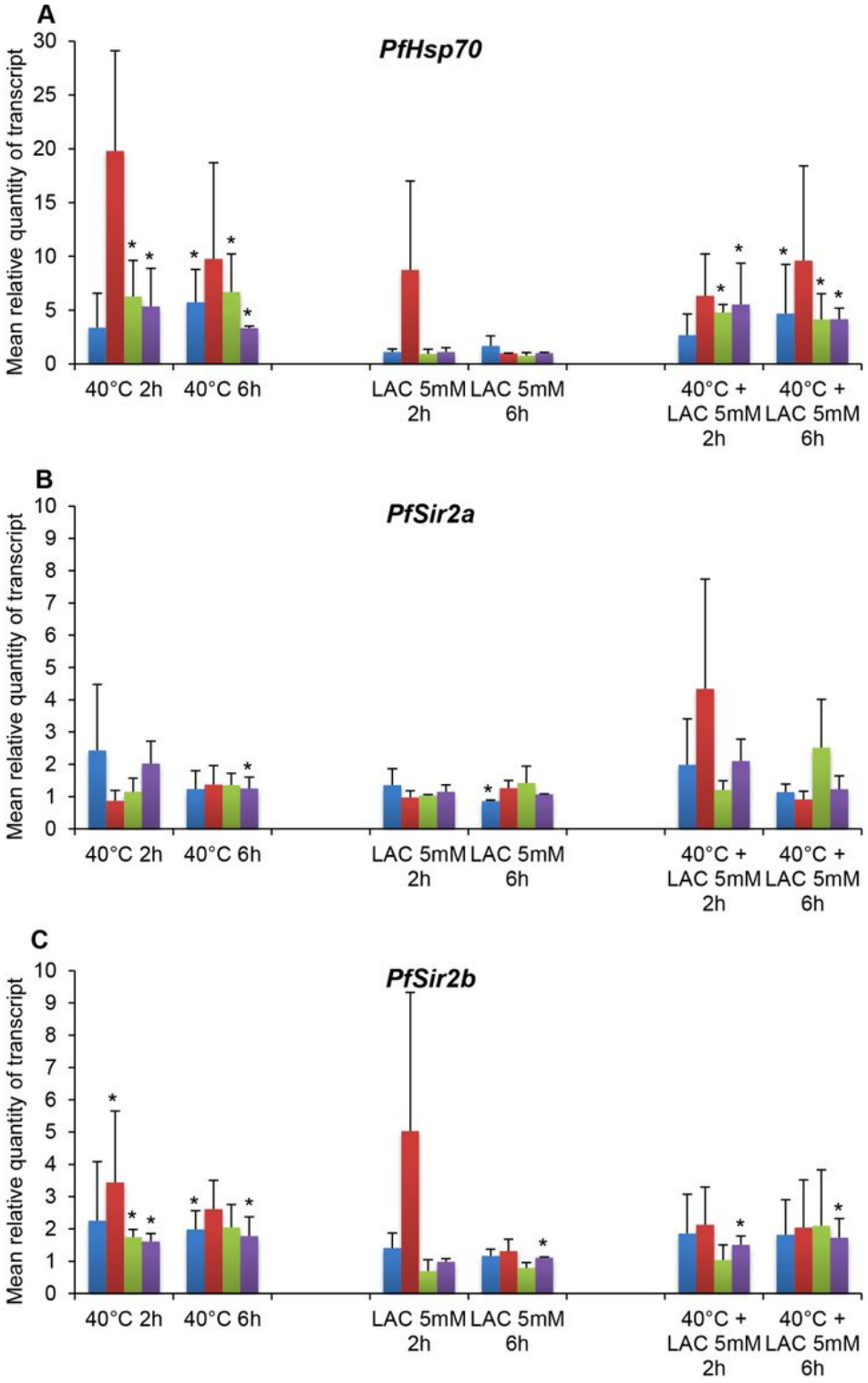

Figure 2

Increased expression of Hsp70, PfSir2a and PfSir2b in response to stress in trophozoites of P. falciparum. Relative quantity of transcripts for Hsp70 (A), Sir2a (B) and Sir2b (C) in parasites after stress treatments,

relative to transcript quantities in a control culture. Blue bars, 3D7; red bars, ID3518; green bars, ID9775; purple bars, ID10668. Mean relative quantity is the mean of RQs from six technical-replicate experiments in strains ID3518, ID9775 and ID10668, or nine technical-replicate experiments in 3D7. Error bars represent standard deviation. *, $p < 0.05$, Kruskal-Wallis test with Dunn's post test.

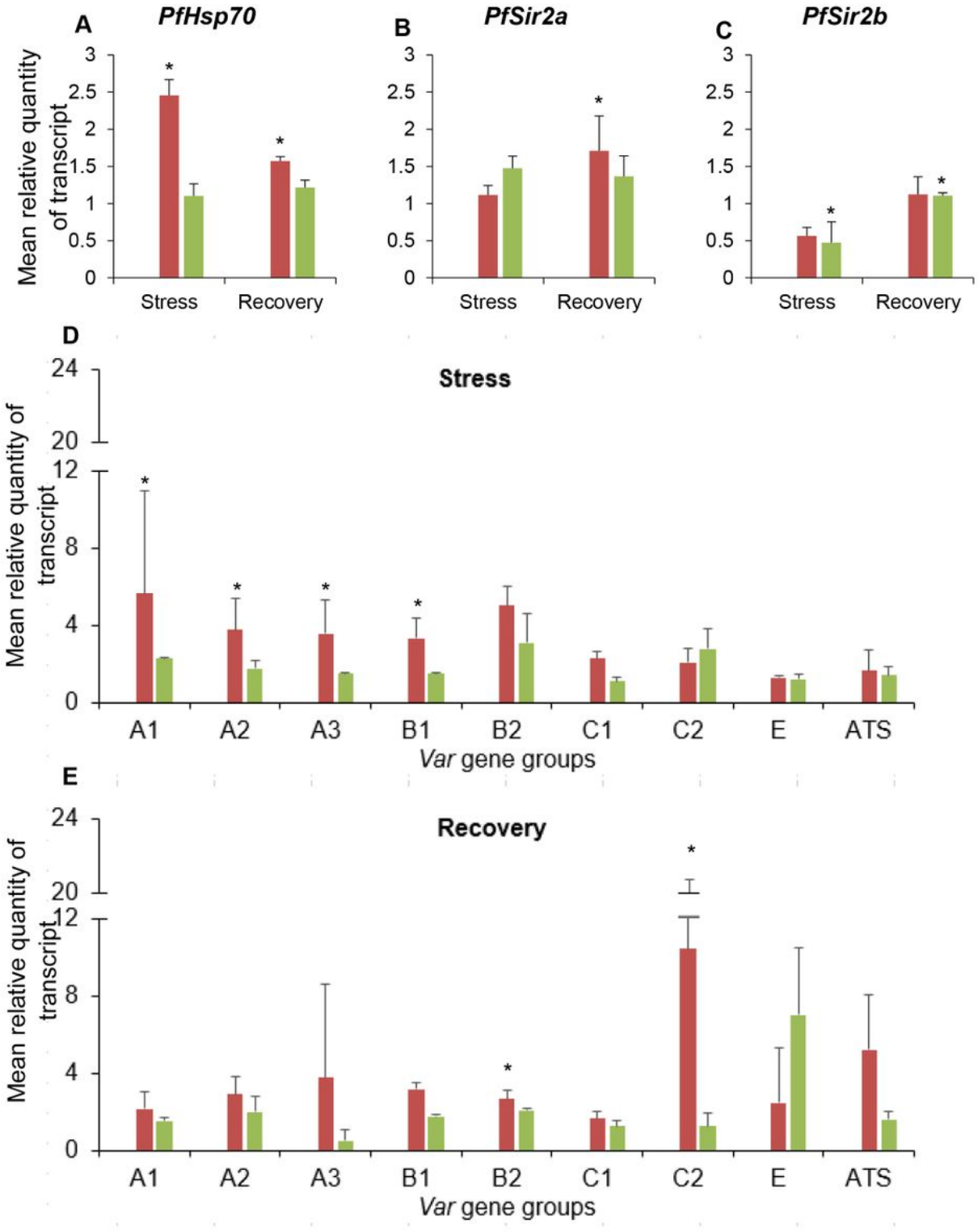

Figure 3

Sirtuin and var gene transcription in ring-staged P. falciparum parasites after heat shock. Relative quantity of transcripts for Hsp70 (A), Sir2a (B) and Sir2b (C) in parasites immediately after 6 h of heat shock ('stress') and after a further 10 h of recovery ('recovery'), relative to transcript quantities in a control culture. Red bars, ID3518; green bars, ID9775. The corresponding relative quantities of var gene group transcripts are depicted in D. immediately after heat shock and E. after 10 h of recovery. Mean relative quantity is the mean RQ from six technical-replicate experiments on each strain. Error bars represent standard deviation. *, $p < 0.05$, Kruskal-Wallis test with Dunn's post test.

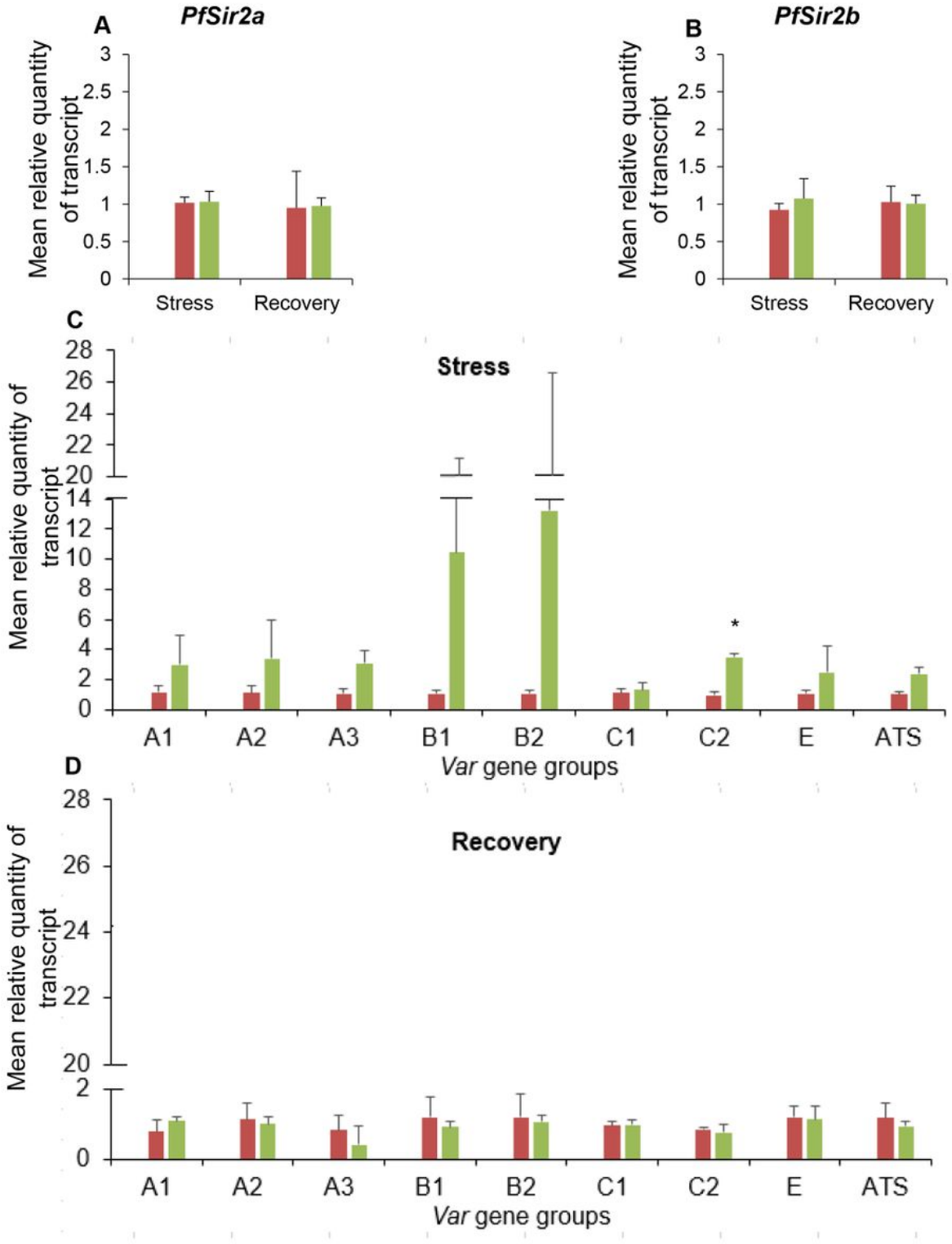

Figure 4

Sirtuin and var gene transcription in ring-staged P. falciparum parasites after exposure to elevated lactate. Relative quantity of transcripts for Sir2a (A) and Sir2b (B) in parasites immediately after 6 h of lactate exposure ('stress') and after a further 10 h of recovery ('recovery'), relative to transcript quantities in a control culture. Red bars, ID3518; green bars, ID9775. The corresponding relative quantities of var gene group transcripts are depicted in C. immediately after lactate exposure and D. after 10 h of recovery. Mean relative quantity is the mean RQ from six technical-replicate experiments on each strain. Error bars represent standard deviation. *, $p < 0.05$, Kruskal-Wallis test with Dunn's post test.

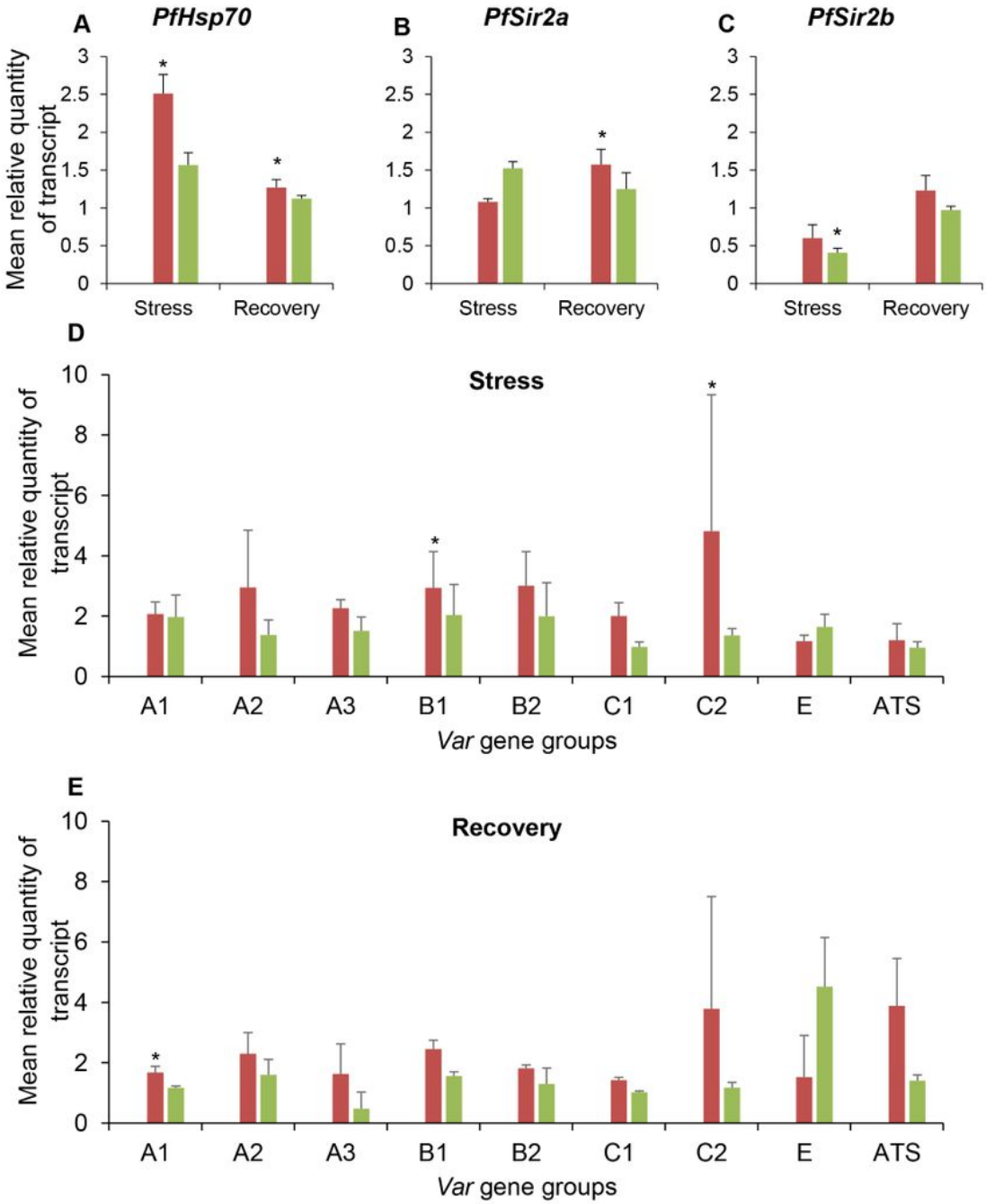

Figure 5

Sirtuin and var gene transcription in ring-staged P. falciparum parasites after heat shock combined with elevated lactate. Relative quantity of transcripts for Hsp70 (A), Sir2a (B) and Sir2b (C) in parasites immediately after 6 h of heat shock and lactate exposure ('stress') and after a further 10 h of recovery ('recovery'), relative to transcript quantities in a control culture. Red bars, ID3518; green bars, ID9775. The corresponding relative quantities of var gene group transcripts are depicted in D. immediately after heat

shock and E. after 10 h of recovery. Mean relative quantity is the mean RQ from six technical-replicate experiments on each strain. Error bars represent standard deviation. *, $p < 0.05$, Kruskal-Wallis test with Dunn's post test.

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