Alteration of the expression of sirtuins and *var* genes by heat shock in the malaria parasite *Plasmodium falciparum*

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Highlights

- *PfSir2B* can be increased in the trophozoite stage in response to heat shock.
- In the ring stages, *PfSir2B* may be downregulated.
- The correlation between high lactate level, and sirtuin and var expression as demonstrated in a field study is not causative.

ABSTRACT

Background: In Plasmodium falciparum the monoallelic expression of *var* virulence genes is regulated through epigenetic mechanisms. A study in the Gambia showed that an increase in *var* gene expression is associated with fever, high blood lactate with commonly-expressed *var* genes expressed in patients with severe malaria. A strong association was demonstrated between the upregulation of *PfSir2A* and group B *var* genes. A subsequent study in Kenya extended this association to show a link between elevated expression of *PfSir2A* and overall *var* transcript levels.

We investigate here the link between heat shock and/or lactate levels on sirtuin and *var* gene expression levels in vitro.

Methods: *In vitro* experiments were conducted using laboratory and recently-laboratory-adapted Kenyan isolates of *P. falciparum*. 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 increase the expression of *PfSir2B* in the trophozoites, whereas exposure to lactate was not. After the ring stages were exposed to heat shock and lactate, there was no alteration in the expression of sirtuins and severe-disease-associated upsA and upsB *var* genes. The association between high blood lactate and sirtuin/*var* gene expression that was previously observed *in vivo* appears to be coincidental rather than causative.

Conclusions: This study demonstrates that heat stress in a laboratory and recently-laboratory-adapted isolates of *P. falciparum* results in a small increase in *PfSir2B* transcripts in the trophozoite stages only. This finding adds to our understanding of how patient factors can influence the outcome of *Plasmodium falciparum* infections.

Keywords: *Plasmodium, var*, antigenic variation, sirtuin, *Sir2A*, *Sir2B*, heat shock

1.0 INTRODUCTION

Several hundred thousand people die from malaria each year. In 2019, 409,000 deaths were recorded. This is almost always due to infection with the malaria parasite specie *P. falciparum*, which can cause severe and lethal malaria. 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 high blood lactate 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)). The host's immune system can recognise these PfEMP1 adhesins (16), however, the parasite has evolved to switch its expression regularly (17,18), allowing immune evasion (19), and subsequently persistent and repeated infections (20,21).

Var genes are grouped into the following groups; A, 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 group A *var* genes, encoding large and complex PfEMP1s that tend to correlate with the severe disease (8–14), are expressed preferentially in immunologically naïve individuals (8). There is some evidence that the upsB group is also associated 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). The mutually exclusive expression means that at any one time, most 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 A, C and E, while PfSir2B primarily controls the group B (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 high blood lactate, 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 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 denote cause-and-effect relationships. Thus, indicating strategies evolved by the parasite to survive heat stress manifesting as elevated temperature following proinflammatory responses and high lactate following hypoxia. *P. falciparum* parasites were accordingly subjected to elevated temperature and high lactate levels in vitro, mimicking the high body temperature and high blood lactate that occur in severe malaria.

2.0 MATERIALS AND METHODS

2.1 Parasite lines and culture

P. falciparum (3D7 (obtained from MR4) and 3 Kenyan strains (obtained from the European Malaria Reagent Repository, 9775, 3518 and 10668)) 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 per the Human Tissue Authority (HTA) license held by Keele University.

2.2 Synchronization of parasites

Double sorbitol synchronization was carried out as described (31), 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 to ensure that synchrony is not lost as the parasites would have to invade new red blood cells and become rings again before the experiments.

2.3 Stress treatment of parasites

Stress treatment was carried out at 4% haematocrit. Trophozoites at 28 ± 4 hours post-invasion (hpi) were subjected to elevated temperature (40°C) and/or to 5 mM sodium lactate for 2 or 6 h. These stressors were chosen to mimic, respectively, the common level of fever in the human host and the WHO threshold for hyperlactataemia that defines severe malaria (2). This level of heat shock was chosen because temperatures above 40°C were previously shown to kill most mature forms of the asexual stage (32) and in our laboratory [unpublished data]. For the experiments with the rings, only two parasite strains, 9775 and 3518 were used. They were exposed to these stressors at 2 ± 2 hpi for 6 h only. This longer time point was used because the stress experiment in the trophozoites indicated that there was little difference in response between the two time points and the *PfSir2B* response was even higher at 6 h compared to 2 h. This combined with the difficulty in extracting enough RNA from young rings informed our decision. 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: total RNA was extracted immediately, and after 10h recovery (at 16 ± 2 hpi 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. The difference in the time to assessment for the two parasite stages is to allow 24 more hours after a whole cycle for the reinvaded rings to develop into trophozoites when the DNA content will be parallel to the parasite numbers. Growth was then measured by SYBR Green 1-based fluorescence assessment as described below.

2.4 SYBR Green 1-based fluorescence assessment

Growth was assessed using the SYBR Green 1-based fluorescence method (33) 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. Survival was calculated using the equation: (gt/gc) x 100, where gt = fluorescence of the test sample and gc = fluorescence of the control sample.

2.5 Gene expression analysis

Total RNA was extracted from each sample culture as described previously (34), but rather than adding Trizol to the iRBCs, Trizol was added directly to the parasite pellet. Briefly, parasites were released from RBCs by saponin lysis at a concentration of 0.1%, (using an equal volume of a 0.2% solution of saponin to the pelleted blood). Free parasites were briefly washed with ice-cold sterile-filtered 1xPBS. Trizol was then added to the parasite pellet, mixed and incubated for 5 mins at 37°C to initiate the RNA extraction. The mixture was stored at - 80°C and the RNA extraction was completed within 2 weeks of storage. RNA yield was quantified using a Nanospec 1000 (Thermo Scientific, USA). Contaminating DNA was removed using the wipeout 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 (35).

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 (a 1:20 dilution at most) 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-staged. 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 (Supplemental file 1, Table S1), their specificity was validated and minimal formation of primer-dimers was

confirmed. Other primer sequences are in Supplemental file 1 (Tables S2 and S3). All primers were manufactured and HPSF®-purified by Eurofins mwg®.

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 $2^{-\Delta Ct}$. ΔCt is the difference between the Ct (threshold cycle value, also known as quantification cycle (Cq), is the number of PCR cycles at which the fluorescence generated by the PCR product is distinguishable from the background fluorescence) of a target gene and the average Ct of the three reference genes, as shown in Supplemental file 2 alongside all raw qPCR data. Log10 of the mean RCN is represented in all the gene expression graphs and was calculated from each biological replicate log10 RCN. Mean fold change in expression was calculated from the difference in the log10 mean RCN of the control and stressed groups by determining the inverse of the antilog of their mean difference.

2.6 Cloning and expression of luciferase reporter gene

In an attempt to validate the absence of any change in *Sir2A* expression after heat shock, we designed a second independent approach (https://dx.doi.org/10.17504/protocols.io.btsxnnfn) to measure this response. The firefly luciferase gene (Fluc) was amplified from an existing expression cassette (36) using primers P5 and P6 (Supplemental file 1, Table S1), and ligated into the pLNSir2GFP plasmid (11) between the AflII and AvrII sites. Specifically, cloning was under the presumptive *Sir2A* promoter (~1.7kb of the gene's upstream sequence) in this plasmid. Cloning was conducted in the PMC103 strain of *E. coli* (ATTC). The resulting plasmid, pLNSir2aproluc (Supplemental file 3, Figure S1A), was transfected into the 3D7 strain as previously described (37). The stress experiments described above were then carried out using the trophozoites of *the PfSir2A-luc* line of 3D7.

2.7 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 for each field strain. 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 values showed no evidence of heteroscedasticity (variances were not significantly different for all groups; the data are spread equally) 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 greater than 10 (38) (Supplemental file 4). Multiple comparisons were done using Turkey's pairwise comparisons across the categorical predictors.

3.0 RESULTS

3.1 Heat shock adversely affects parasite survival whereas lactate exposure does not

To choose the 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 the trophozoite stage.

48 h after sub-culturing the young trophozoites stressed at 28 ± 4 hpi, heat shock at 40°C resulted in 0 - 23% death following a 2 h exposure and 15 - 43% death following a 6 h exposure (Fig. 1A). The extent of heat-shock-induced death appeared strain-dependent, with one strain (9775) having a higher sensitivity as there was a 23% and 43% death following 2 and 6 h of stress respectively (Fig. 1A, green bar). The heat-shock-induced death in the young rings stressed for 6 h at 2 ± 2 hpi, as calculated from the SYBR Green 1-based fluorescence values 72 h post-stress (Fig. 1B) ranged from 3 - 27% like the trophozoites exposed to heat shock at 40°C for 2 h only thus indicating that the rings are expectedly less sensitive (39). Strain 9775 was equally more sensitive here with 27% death.

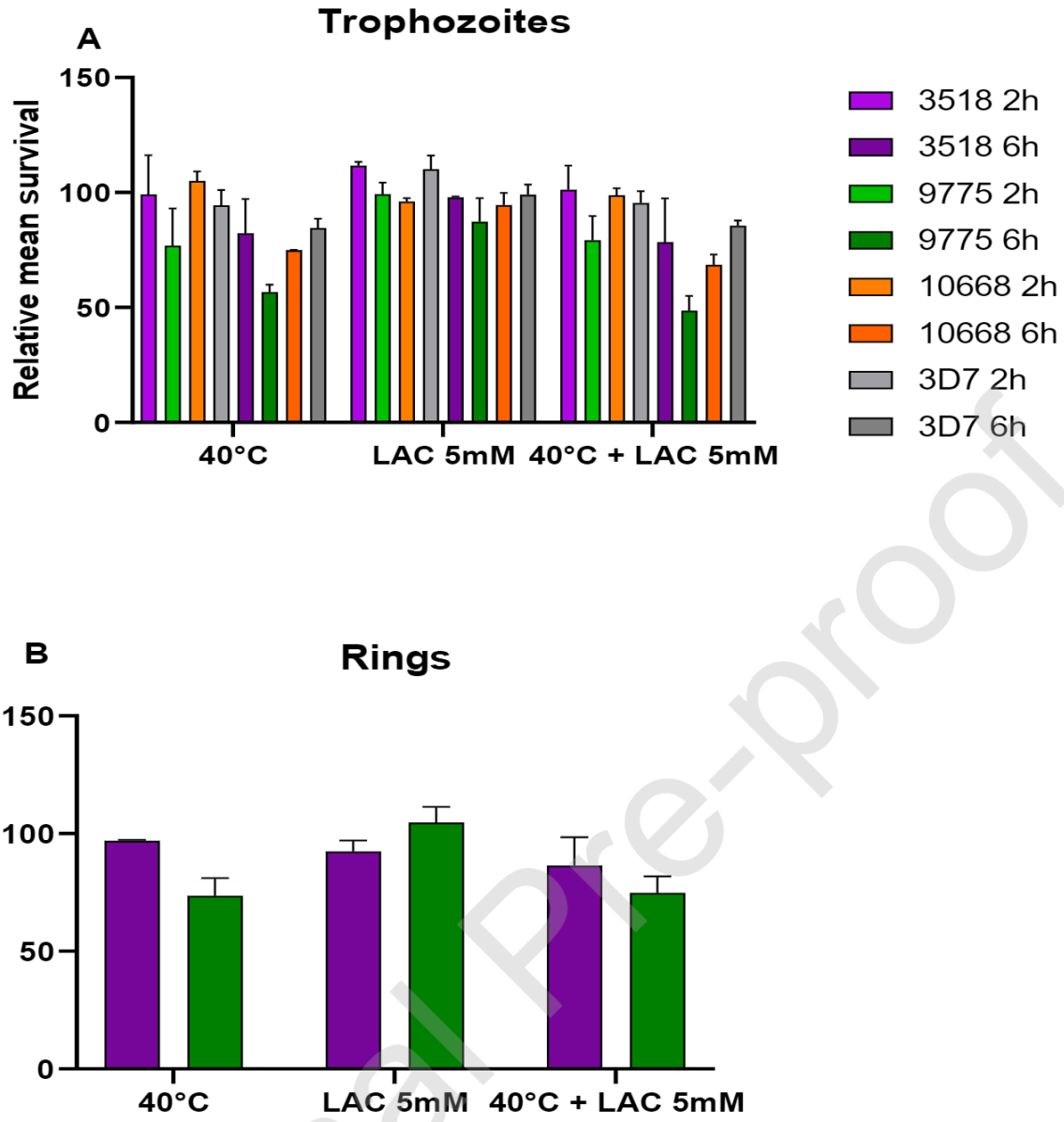

Fig. 1: Post-stress survival of *P. falciparum* after exposure to lactate and heat shock. (colours should be used in print)

A. Parasite survival 48 h after stress treatment for 2 h or 6 h at the trophozoite stage, relative to the 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 the growth of a control culture, measured via DNA content in a SYBR-Green-1 based assay. Blue bars, 3D7; red bars, 3518; green bars, 9775; purple bars, 10668. Mean survival rates are from biological duplicates for the Kenyan strains or triplicates for 3D7. Error bars represent standard error. No statistical analysis was performed as n was small, ≤ 3.

By contrast to heat shock, lactate exposure had little or no adverse effect on the survival of the young trophozoites with a 1 - 4% death after exposure for 2 h and a 1 - 12% death after exposure for 6 h (Fig.1A). In strains 3D7 and 3518 there was up to a 10% growth increase compared to the control after 2 h of stress. Lactate exposure for 6 h at the ring stage again had a negligible effect on parasite survival and resulted in a 0 – 7% death (Fig. 1 B). In the Kenyan strain 9775, there was a 4% increase in growth. Survival for both parasite stages was similar after exposure to lactate, notwithstanding that the trophozoite is the most metabolically active stage, at which lactate production is highest. When both stressors were applied together, results were remarkably like those seen after heat shock alone (Fig. 1A & B).

3.2 Alteration of hsp70, Sir2A and Sir2B expression in heat-shocked trophozoite and ring stages of P. falciparum

Having established the heat shock conditions that caused only a moderate amount of parasite death in both trophozoites and rings, the expression of sirtuin genes immediately after heat shock was measured, as well as the expression of the gene encoding heat shock protein 70 (*hsp70*) as a positive control (Fig. 2 and Table 1). In the trophozoite stage, *hsp70* expression was upregulated (Fig. 2A) by 4.70 and 4.92 folds after 6 and 2 h of heat shock respectively (Table 1) or by 3.42 and 3.69 folds after 6 and 2 h of heat shock combined with lactate, respectively. Expectedly, there was no increase in *hsp70* after lactate exposure.

PfSir2A expression did not change significantly in either trophozoites or rings under any of the conditions tested as it remained the same when time, temperature or treatment factors were used to compare its expression (Fig. 2C, Table 1). *PfSir2B* expression was increased after 2 h and 6 h of heat shock (Fig. 2E) with mean fold changes of 1.88 and 1.98, respectively (Table 1). Like the expression of *hsp70*, there was also an increase in *PfSir2B* after heat shock was combined with lactate with a 1.45 and 1.48-fold change after 2 h and 6 h exposure, respectively. 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*.

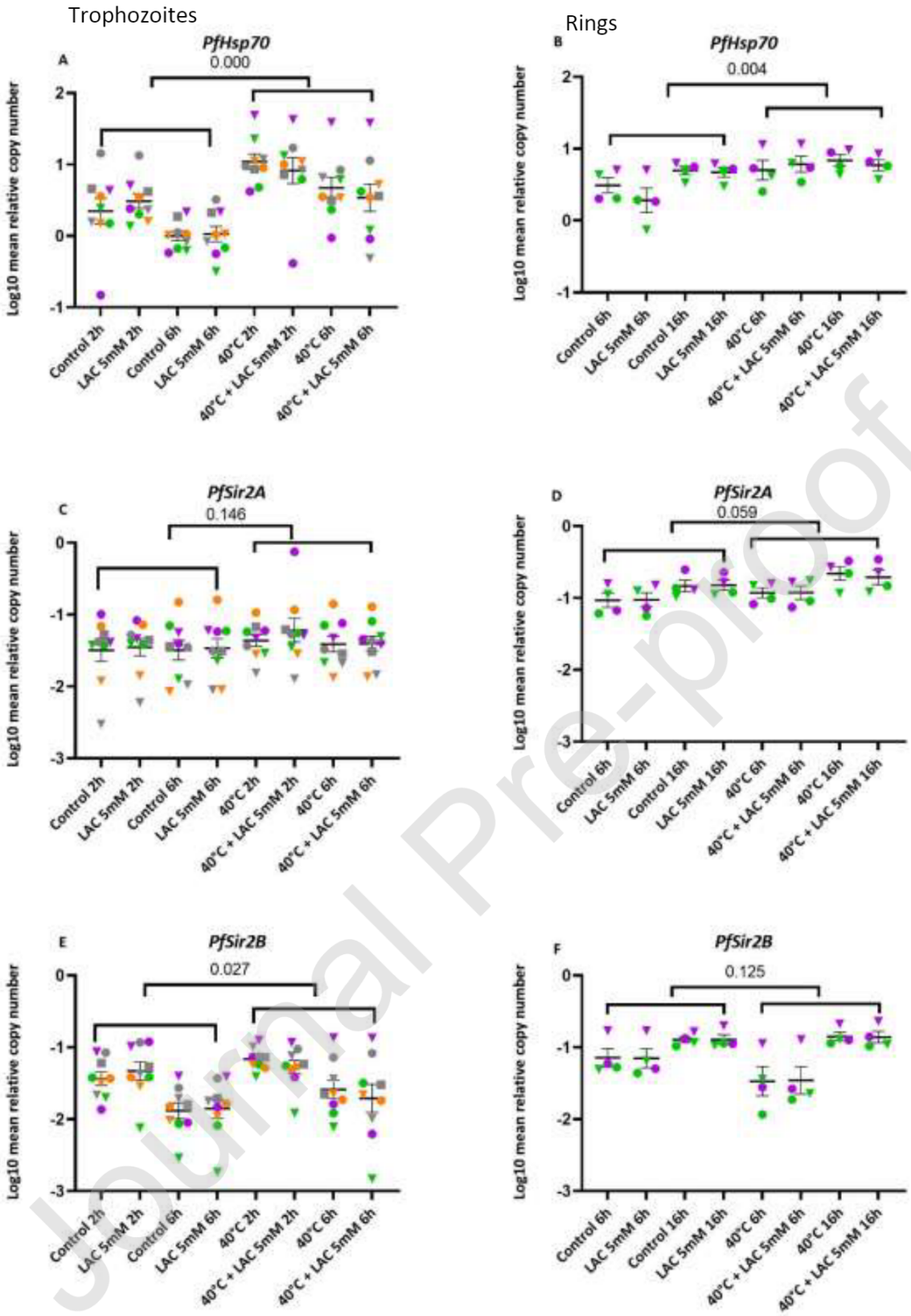

Fig. 2: Altered expression of *Hsp70* and *PfSir2B* in response to heat shock in *P. falciparum*. (colours should be used in print) (Legend on the next page).

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, 3518, 9775 and 10668, respectively. Circles, inverted triangles and squares are data from the first, second and third biological replicate experiments, respectively. Error bars show standard error mean (SEM). Significance was determined by fitting a general linear model with Turkey's pairwise comparisons across the categorical predictor of temperature (37° or 40°C). In the trophozoites, p = 0.000 for *Hsp70*, p = 0.146 for *Sir2A* and p = 0.027 for *Sir2B*, while in the rings, p = 0.004 for *Hsp70*, p = 0.059 for *Sir2A* and p = 0.125 for *Sir2B*.

Table 1

Fold changes in the expression of *hsp70*, *Sir2A* and *Sir2B* in trophozoites after stress

Gene/Time of exposure	**Lactate 5mM**	**40°C**	**40°C + Lactate 5mM**
Hsp70 2h	1.39 (0.49 to 3.89)	4.92 (1.73 to 14.00)	3.69 (1.06 to 12.88)
Hsp70 6h	1.06 (0.57 to 1.99)	4.70 (2.06 to 10.72)	3.42 (1.20 to 9.76)
Sir2A 2h	1.11 (0.43 to 2.87)	1.37 (0.57 to 3.26)	1.90 (0.64 to 5.64)
Sir2A 6h	1.06 (0.42 to 2.73)	1.21 (0.52 to 2.87)	1.23 (0.53 to 2.88)
Sir2B 2h	1.28 (0.59 to 2.76)	1.88 (1.11 to 3.20)	1.45 (0.76 to 2.77)
Sir2B 6h	1.07 (0.46 to 2.49)	1.98 (0.86 to 4.54)	1.48 (0.49 to 4.57)

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. The calculation of mean fold changes is described in the gene expression analysis subsection in the methods.

The expression of the sirtuins was equally 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) (40). Only two strains (9775 and 3518) were selected for these experiments