Minor temperature shifts do not affect chromosomal ploidy but cause transcriptomic changes in *Leishmania braziliensis* promastigotes *in vitro*

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BACKGROUND The leishmaniases are complex neglected diseases caused by protozoan parasites of the genus *Leishmania*. *Leishmania braziliensis* is the main etiological agent of cutaneous leishmaniasis in the New World. In recent studies, genomic changes such as chromosome and gene copy number variations (CNVs), as well as transcriptomic changes have been highlighted as mechanisms used by *Leishmania* species to adapt to stress situations.

OBJECTIVES The aim of this study was to determine the effect of short-term minor temperature shifts in the genomic and transcriptomic responses of *L. braziliensis* promastigotes *in vitro*.

METHODS Growth curves, genome and transcriptome sequencing of *L. braziliensis* promastigotes were conducted from cultures exposed to three different temperatures (24°C, 28°C and 30°C) compared with the control temperature (26°C).

FINDINGS Our results showed a decrease in *L. braziliensis* proliferation at 30°C, with around 3% of the genes showing CNVs at each temperature, and transcriptomic changes in genes encoding amastin surface-like proteins, heat shock proteins and transport proteins, which may indicate a direct response to temperature stress.

MAIN CONCLUSIONS This study provides evidence that *L. braziliensis* promastigotes exhibit a decrease in cell density, and noticeable changes in the transcriptomic profiles. However, there were not perceptible changes at chromosome CNVs and only ~3% of the genes changed their copies in each treatment.

Key words: *Leishmania braziliensis* - temperature shift - copy number variation - transcriptome profile - heat shock protein - amastin-like protein - ABC transporter

Leishmaniases are caused by protozoan parasites of the *Leishmania* genus and involve a variety of clinical manifestations: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis and visceral leishmaniasis. These diseases are a major public health problem in 98 countries around the world, where 12 million people are infected, more than 350 million people are at risk of infection and 1.3 million new cases occur each year. (1) CL is the most common clinical manifestation; between 0.7 and 1.3 million new cases of CL are reported annually and about 90% of them occur in Afghanistan, Algeria, Brazil, Iran, Pakistan, Peru, Saudi Arabia, Syria and Colombia. CL is also the most important and common clinical manifestation in the New World; it is characterised by ulcerative and deformative lesions, for which the most common causative species in the New World is *Leishmania braziliensis*. (1,2)

Temperature is a factor that determines whether the *Leishmania* parasite can develop appropriately. This was reported in a study by Hlavacova et al. (3) in which they evaluated the effect of temperature on the life cycle of three *Leishmania* species inside two different species of vector (sandflies) and concluded that the response to temperature is species-specific. Other studies reported different results depending on the temperature shifts and the *Leishmania* species studied, some of the results showed an increase in parasite division, morphological changes (the parasites acquired a round shape), loss of motility, (4) and changes in their differentiation rate or survival. (4) However, there have been few studies aimed at understanding the biology of *Leishmania* when subjected to temperature stress. The majority of studies have focused on the impact of temperature shifts on the distribution of the vectors and the corresponding association with the number of leishmaniasis cases. (5)

Other studies have evaluated this variable in the several *Leishmania* species promastigotes in terms of the thermal shock associated with a change of host, in which the specific production of heat shock proteins (HSPs) occurs with an increase in temperature. (6,7,8) However, little is known about the effect of temperature on the genomic and transcriptomic responses of *Leishmania*.

However, other studies on *Leishmania* have provided insights into the important role that genotypic plasticity plays in the response to different stress situations, with chromosome ploidy changes, amplifications and/or deletions of whole genes or chromosomes [copy number variations (CNVs)], being some of the mechanisms used
by Leishmania to acquire resistance to antileishmanial drugs, to adapt to environmental conditions, or to distinguish different strains and species. Furthermore, some studies reported the impact of genomic changes on the transcriptome of Leishmania. For example, Dumetz et al. revealed the correlation between some chromosomal CNVs with the transcriptomic profile of the parasite, and Iantorno et al. showed that 85% of the differences in gene expression among L. tropica isolates could be explained by CNVs at the chromosomal and gene levels.

Despite awareness that Leishmania genotypic plasticity is an adaptation strategy to stress situations, the impact of temperature shifts on the genome and/or transcriptome of Leishmania parasites is poorly understood. In this study, we used next generation sequencing (DNA-seq and RNA-seq) to determine the possible genomic and transcriptomic impacts that shifts in temperature may have on L. braziliensis promastigotes in the short-term in vitro. Our findings provide the first insights into the genomic and transcriptomic changes that might confer the short-term adaptive capacity of Leishmania to temperature stress, increasing awareness of the biology of this important parasite in the New World.

MATERIALS AND METHODS

Culture conditions and growth curves - Promastigotes of L. braziliensis from strain MHOM/BR75/M2904 were cultivated in Roswell Park Memorial Institute (RPMI) (Sigma-Aldrich, MO, US) growth medium supplemented with 10% heat-inactivated foetal bovine serum (Invitrogen, CA, US). The parasites were incubated at four different temperatures, 24°C, 26°C, 28°C and 30°C, and the cultures at each of these temperatures were incubated with 5% CO₂. A control temperature of 26°C was included because it is a standard temperature for in vitro promastigote culture. We prepared cultures with three biological replicates per temperature with an initial concentration of 1 × 10⁶ parasites/mL. The parasite density for all culture replicates at each temperature was quantified using a Neubauer chamber for seven consecutive days. This quantification was used to construct growth curves for each temperature. From these curves, we determined the beginning of the logarithmic phase (BLP) by defining the day on which a significant difference emerged compared with the initial concentration. Statistical analyses were performed using the program GraphPad Prism (https://www.graphpad.com/scientific-software/prism/), where we analysed normality using the Kolmogorov–Smirnov test and subsequently used the Dunnett test of multiple comparisons to determine the day on which the logarithmic phase started. To determine whether there was a difference between the evaluated temperatures, we conducted a two-way ANOVA test comparing each treatment with the control temperature. In addition, we performed a Kruskal–Wallis test to determine whether the parasite density during the extraction day was significantly different between the treatments. P values < 0.05 were considered statistically significant.

Isolation of RNA and DNA - DNA and RNA extractions were performed on the day of the BLP, as defined by the parasite growth curves at each temperature. The DNA was extracted from one sample per treatment and was isolated using the Ultraclean Tissue and Cell DNA Isolation kit (MO BIO, CA, US), following the manufacturer’s standard protocol. Total RNA was extracted from two independent biological replicates, using the RNasey Plus Mini Kit (Qiagen) following the manufacturer’s instructions. To control the sequencing process reliability, two technical replicates from each biological replicate were included. The concentration and quality of the DNA and RNA samples were quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific™), and the integrity was assessed by electrophoresis in a 1% agarose gel. All samples had A₂₆₀/A₂₈₀ ratios higher than 2.0. See Supplementary data (Fig. 1) for the integrity data and the A₂₆₀/A₂₈₀ values of all of the samples.

Genome and transcriptome sequencing - Once extracted, the DNA and RNA were sequenced by the Illumina HiSeq X-TEN system and libraries were prepared as follows: Microbial Mate-Paired for DNA and Strand-specific TrueSeq RNA-seq Library Prep (Illumina) for RNA with an insert size of 350 bp. The reads were 2 × 150 bp in length. The sequencing was performed by Novogene Bioinformatics Technology Co., Ltd., Beijing, China. The software FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to determine the reliability of the sequencing.

Mapping of DNA and RNA reads - The reads were mapped to the reference genome L. braziliensis MHOM BR75 M2904 using the software SMALT v0.7.4 (www.sanger.ac.uk/resources/software/smalt/) with an exhaustive searching option -x and -y 0.8, a reference hash index of 13 bases and a sliding step of 3. We also mapped the reads with an identity threshold y = 0.8 to prevent mapping of non-Leishmania reads to the reference. Finally, read file merging, sorting and elimination of PCR duplicates were implemented with the software Samtools v0.1.18 (https://sourceforge.net/projects/samtools/) and Picard v1.85 (https://broadinstitute.github.io/picard/).

Genomic data analysis

Evaluation of chromosome and gene copy number variations (CNVs) - To obtain the read depth per chromosome, the sequencing data were normalised by the mean depth for the 35 chromosomes of L. braziliensis. The range of chromosome ploidy (P) was determined from the normalised read depths of the chromosomes and was defined as follows: p < 1.5 (haploid), 1.5 ≤ p < 2.5 (diploid), 2.5 ≤ p < 3.5 (triploid), 3.5 ≤ p < 4.5 (tetraploid) and 4.5 ≤ p ≤ 5.5 (pentaploid). Also, we used vcf tools for calculating allelic chromosome frequency to evaluate and confirm ploidy results. The heatmaps were created using the R package Heatmap3. We compared the ploidy between temperatures and against control temperatures by calculating the p value using a Kruskal-Wallis test for independent samples.

To evaluate gene CNVs, the mean read per gene was determined, considering the possible impact of chromosome ploidy. The genes were filtered using the thresholds of a fold-change of Z score > 2 and p value < 0.05 compared with the control temperature of 26°C. Then, the filtered genes were analysed according to the shared
and unique genes identified at each temperature. For the supplementary figures of the read depth distribution of important chromosomes, we first calculated the depth with Samtools and then plotted the data with the program Gnuplot (http://www.gnuplot.info/). Data were classified into genomic locations and gene ontology (GO) terms were obtained through the free database TriTrypdb (http://tritrypdb.org/tritrypdb/), and the software Revigo was used to summarise the GO terms and remove any redundancy. We calculated the percentage of genes per ontology classification considering the total of genes with CNVs (i.e., with an increased or decreased copy number). We used a cut-off of > 5% of genes categorised to a specific GO term and all of the ontology terms had a \( p \) value of \(< 0.05\). Statistical analysis was performed using the software GraphPad Prism. We performed tests of normality using the Kolmogorov–Smirnov and Shapiro-Wilk tests and then the Kruskal-Wallis test as a nonparametric test for independent samples to compare the read depths of each gene following different temperature shifts. For the GO analysis, we used terms incorporating a high percentage of genes; the percentages were calculated from the total of genes with CNVs (i.e., an increased or decreased copy number) compared with the control temperature. Finally, we constructed Venn diagrams, using Microsoft Office tools, taking into consideration the quantity of shared and unique genes with CNVs across the three temperatures.

**Single-nucleotide polymorphism (SNP) analysis** - To conduct single-nucleotide analysis, the SNPs, small insertions and deletions were called by the software Toolkit v3.4 GATK (https://software.broadinstitute.org/gatk/). Low-quality SNPs were filtered using GATK Variant Filtration, and Samtools was used to avoid false positives. The software SnpEff v4.1 (http://snpeff.sourceforge.net/) was used to classify the indels and SNPs according to their impact, such as being synonymous or nonsynonymous, or having a high or moderate impact in the genome.

**Transcriptome data analysis** - The levels of transcripts were quantified by assessing the read depth. The relative RNA-based ploidy (RNA-P) per chromosome was computed using the average read depth of transcripts and heatmaps were created using the R package Heatmap3. The ploidy range was the same as described previously in the DNA analysis section (2.5.1) and was based on the study by Rogers et al.(13) The obtained results were statistically analysed through two-way analysis of variance (ANOVA) (software GraphPad Prism) to determine whether each treatment was associated with a significant change in RNA compared with the results at 26°C. To assess the impact of the gene CNVs in the transcriptomes, we calculated Spearman's correlation coefficient using the software GraphPad Prism.

Differentially expressed genes (DEGs) were identified using DESeq 1.18.1 (R/Bioconductor); we used a fold-change cut-off of \( > 2 \) and a \( p \) value \(< 0.05\) to define DEGs. The proportion of DEGs per chromosome was defined as follows: (number of DEGs per chromosome) / (number of total genes per chromosome) \( \times 100\). For the supplementary figure showing the read depth distribution of chromosomes with the highest number of up- or down-regulated genes, we first calculated the depth with Samtools and then plotted the data using the program Gnuplot (http://www.gnuplot.info/). Finally, GO information was extracted from a database (http://tritrypdb.org/tritrypdb/) using the option ‘biological process’ and a \( p \) value \(< 0.05\) for the classification. We used Revigo software to summarise the GO terms and removed any redundancy. The Venn diagrams and GO figures were constructed using Microsoft Office tools, the GO analysis and its representation was performed through calculation of the percentage of genes associated with each GO term considering the total number of DEGs for each classification (up- and downregulated genes).

**RESULTS**

**Growth curves of L. braziliensis promastigotes incubated in vitro under different temperatures** - At the control temperature, promastigotes reached the BLP on day 6, and no decrease in the cell density was observed in the control cultures (26°C) over the course of the experiment (Fig. 1). Whereas at 24°C, promastigotes reached the BLP on day 3, and similarly no decrease in the cell density was observed. After day 3, the number of parasites increased only slightly during the rest of the experiment (Fig. 1). The growth curves of the promastigotes at 28°C and 30°C were similar; in both cases, the BLP was on day 2, followed by a decrease in the cell density on the last days of the experiment (Fig. 1). Despite the similarity in pattern between these two highest temperatures, the magnitude of the cell density differed between them, with fewer parasites being observed at 30°C (Fig. 1). Notably, the growth curves differed significantly at the different temperatures (\( p \) < 0.05). RNA and DNA extractions were performed on the BLP at each temperature, i.e., day 6 at 26°C, day 3 at 24°C, and day 2 at 28°C and 30°C.

**Evaluation of copy number variations at the chromosomal level** - From the DNA sequencing data [the DNA-seq statistics are summarised in Supplementary data]...
(Table I), a comparison was made of the chromosomal ploidy of the samples at different temperatures (24ºC, 26ºC, 28ºC and 30ºC), which is illustrated in a heatmap in Fig. 2A. No significant difference in DNA ploidy was detected among the four temperatures evaluated (p = 0.975). This indicated that there was no change in ploidy of *L. braziliensis* promastigotes due to exposure to different temperatures over the short-term. In summary, three chromosomes (3, 16 and 24) were found to be disomic; chromosomes 4, 6 and 27 were tetrasomic; and chromosome 31 was pentasomic. The remaining 28 chromosomes were trisomic, as expected.

To confirm the somy results, we calculated the allele frequency finding similar results to those obtained by mean normalisation depth. For example, the chromosome 7 is trisomic showing frequencies of 0.66 and 0.33; chromosome 27 is tetrasomic with allele frequency of 0.25 - 0.5 and 0.75, and chromosome 24 is disomic showing a frequency around 0.5; see Supplementary data (Fig. 2).

Moreover, for each chromosome, we computed the mean transcript level to determine the ploidy value based on the results of RNA sequencing (RNA-P). Then, we compared the RNA-P with the DNA ploidy, as described above. We did not observe a difference in ploidy between the control and the treatments in any of the chromosomes (Fig. 2A). We also observed no difference in ploidy based on RNA-P (Fig. 2B). When comparing the control with each treatment using two-way ANOVA, the following p values were obtained: 0.073 (control (26ºC) vs. 24ºC), 0.220 (control vs. 28ºC) and 0.144 (control vs. 30ºC).

**Evaluation of the copy number variations (CNVs) at the gene level** - Gene CNVs were evaluated by comparing the results obtained at each tested temperature (24ºC, 28ºC and 30ºC) with those obtained at the control temperature (26ºC). From a total of 8507 genes, 253 genes (2.97%) at 24ºC, 247 genes (2.9%) at 28ºC and 260 genes (3.05%) at 30ºC presented CNVs (-2 > Z score > 2, equivalent to p < 0.05). While 61.33% ± 1.5% of the genes with CNVs at each temperature showed an increase, the remaining 38.67% ± 1.5% showed a decrease compared with the control. Furthermore, mean values of 41.84% ± 1.7% of the genes with an increased copy number and 32.6% ± 3.6% of the genes with a decreased copy number encoded hypothetical proteins at each temperature assessed.

Supplementary data (Fig. 3) shows the distribution of genes with a fold-change of 2 in CNVs for each temperature compared with the level for the control temperature (26ºC). For all temperatures, the chromosomes with more...
than 10 genes with CNVs were chromosomes 14, 20.1, 27, 31 and 35 (Fig. 3A). The read depth distributions per chromosome showing the changes per position are presented in Supplementary data (Figs 4, 5, 6), and chromosome 31 possessed the highest number of genes with CNVs across the three temperatures [Supplementary data (Tables II–IV)]. In terms of the percentage of genes showing CNVs per chromosome, the highest rate was detected for chromosome 2. In this chromosome, all of the genes with CNVs at 24°C showed an increase in their copy number compared with the control temperature.

Among the three tested temperatures, 92 genes shared CNVs compared with the control (Fig. 3B), and the variations in these genes (i.e., increased or decreased copy number) were consistent across the temperatures. There was also no significant difference in the read depth of the shared genes (p = 0.971 for the genes with increased read depth and p = 0.982 for the genes with decreased read depth). Among the shared genes [Supplementary data (Fig. 7)] that increased in copy number compared with the control were genes that encode elongation factor 1-alpha (LbrM.17.0090) and a surface antigen protein (LbrM.04.1330). Those with decreased copy number included genes that encode an amastin-like surface protein and a NADH-dependent fumarate reductase (LbrM.34.1110). Moreover, some genes that encode amastin-like proteins (LbrM.08.1060, LbrM.18.0460) and beta and alpha tubulins increased their copy number, while others that encode similar proteins decreased their copy number (LbrM.13.0200, LbrM.33.0920, LbrM.33.0950). The genes for which variations were only shared between two temperatures are presented in Supplementary data (Fig. 8). At 28°C and 30°C, more genes showed a decrease in copy number and these included genes that encode a ubiquitin-conjugating enzyme E2 (LbrM.02.0420), tRNAs (LbrM.20.1.tRNA8, LbrM.23.tRNA10, LbrM.23.tRNA6, LbrM.23.tRNA8) and an amastin surface protein (LbrM.24.1590). At 24°C and 28°C, more genes showed an increase in copy number, such as those encoding a peptidase M20/M25/M40 (LbrM.33.2100) and a receptor-type adenylate cyclase (LbrM.17.0110). Finally, between 24°C and 30°C, fewer genes were shared, but the genes that encode HSP83-1 and HSP70 (LbrM.33.0330, LbrM.28.2970) showed a decreased copy number. The number of unique genes totalled 114 genes for 30°C, 102 genes for 24°C and 92 genes for 28°C (Fig. 3B). For these genes, we obtained the associated ontology terms through the database triTrypdb.org, choosing the option ‘biological process’ to determine the function of the genes.

Fig. 4 shows the ontology terms associated with large numbers of genes, all of the GO terms had a p value < 0.05. Some ontology terms were shared between unique genes with an increased copy number at each tempera-
tecture. For example, genes with CNVs at 24°C and 28°C shared the ontology term ‘biological process’ (Fig. 4A), whereas those with CNVs at 30°C and 28°C shared ontology terms such as ‘biological regulation’ and ‘regulation of biological process’ (Fig. 4A). Numerous unique ontology terms were associated with genes with CNVs at 28°C including ‘phosphorylation’, ‘regulation of cellular process’, ‘organonitrogen compound biosynthetic process’ and ‘cellular nitrogen compound metabolic process’ (Fig. 4A). Otherwise, the ontology terms for genes that showed a decreased copy number differed from those associated with genes showing an increased copy number. Some ontology terms that were shared among the three treatments for genes with decreased copy numbers were ‘amide and peptide metabolic and biosynthetic process’ and ‘organonitrogen compound biosynthetic process’ (Fig. 4A). The ontology terms only shared by genes following treatment at 30°C and 24°C were ‘organic substance metabolic process’, ‘cellular biosynthetic process’ and ‘biosynthetic processes’, and the unique terms at 24°C were related to metabolic, cellular and biosynthetic processes (Fig. 4B).

Next, we investigated the genes associated with the ontologies described above. Fig. 5 shows the genes with an increase in copy number compared with that at the control temperature. At 24°C, the gene with the highest read depth encoded HSP70, putative protein (LbrM.28.2990), followed by an iron/zinc transporter-like protein (LbrM.31.3480). At 28°C, one gene with an increased read depth was that encoding GP63 leishmanolysin (LbrM.10.1710) and one gene with a decreased read depth was heat shock 70-related protein 1 (LbrM.30.2420). At 30°C, the notable genes showing an increased copy number were heat shock protein 83-1 (LbrM.33.0340) and beta-tubulin (LbrM.33.0930). By contrast, genes showing a decreased copy number encoded a serine-threonine dehydratase (LbrM.06.0720) and translation elongation factor 1-beta (LbrM.35.1570).

Finally, we evaluated a total of 38,703 SNPs and 44 (0.11%) had a high impact and 7611 (17.33%) had a moderate impact on DNA. Among the three temperatures evaluated, there were no significant differences in the SNPs (p = 0.947). We also evaluated a total of 18,755 indels, and these indels did not differ significantly among the three temperatures (p = 0.939).

**Evaluation of copy number variations (CNVs) at the gene level and the impact on the transcriptome profiles** - After assessing the changes in gene copy number, we evaluated their impact at the transcriptome level. First, to establish whether there was a correlation between the increase/decrease in copy number and the increase/decrease in gene expression, we calculated Spearman’s correlation coefficient. The results indicated no correlation between CNVs and gene expression in all of the treatments, since the r values were near zero and the p values were > 0.05. One example of this lack of correlation was on chromosome 14 at 24°C and 28°C, in which all of the genes with CNVs showed an increase in their copy number, but some
genes such as LbrM.14.1110 and LbrM.14.1310 showed a decrease in RNA synthesis compared with the control. Despite this, we examined the genes with an increase in both their copy number and expression, as well as those with a decrease in their copy number and expression, revealing 15 and 13 genes at 24ºC, 9 and 14 genes at 28ºC, and 11 and 21 genes at 30ºC, respectively.

Interestingly, a gene encoding an elongation factor (LbrM.17.0090) showed an increase in copy number and expression under the three temperatures. Furthermore, we found other genes that encode a hypothetical protein (LbrM.04.0230) and an alpha-tubulin (LbrM.29.2700) that increased their copy number and expression at 24ºC and 28ºC. By contrast, some genes decreased their copy number and expression under the three treatments: two encoding amastin-like surface proteins (LbrM.18.0460 and LbrM.180470), which are also tandem genes, and one encoding an NADH-dependent fumarate reductase gene (LbrM.34.1110). At 24ºC and 30ºC, another gene that encodes an NADH-dependent fumarate reductase gene (LbrM.34.1110) exhibited a decreased copy number and expression; at 28ºC and 30ºC, genes that encode amastin-like surface protein (LbrM.24.1590) and a beta-tubulin (LbrM.33.0990) also showed these decreases; whereas at 24ºC and 28ºC, only one gene that encodes a poly-zinc finger protein (LbrM.35.1790) shared this behaviour. Here, it is worth noting that, at 30ºC, there were two genes of particular interest that could have changed their expression as a direct response to the temperature.

One of these genes encodes a stress-inducible protein (LbrM.35.0120), which increased its copy number and expression; while the other encodes a multidrug resistance protein (LbrM.35.1520), which showed decreases in both its copy number and expression.

**Differentially expressed genes (DEGs) in promastigotes at each assessed temperature** - We evaluated the changes in the transcription profiles of four replicates, two biological and two technical, in response to the different temperatures at which L. braziliensis promastigotes were incubated. The total number of reads obtained for the RNA sequences, as the number of aligned reads per treatment, are represented in Supplementary data (Table V). We compared the gene expression levels with the results obtained at the control temperature (26ºC), obtaining log-fold changes. The cut-off was chosen as a fold change (FC) > 2 (log-fold change > 1), as indicated by the dotted line in Fig. 6. At the three temperatures, similar patterns of DEGs were identified, with all of them including more downregulated genes than upregulated genes (Fig. 6). The promastigotes incubated at 28ºC had 920 genes with FC > 2 and p < 0.05 (11.2%) from a total of 8204 DEGs; of these 920 genes, 639 were downregulated and 281 were upregulated. By contrast, the promastigotes incubated at 24ºC had 1064 genes with FC > 2 and p < 0.05 (13%), from a total of 8205 DEGs; of these 1064 genes, 639 were downregulated and 281 were upregulated. Finally, in order of lowest to highest number of DEGs, at 30ºC the promastigotes
showed changes in the expression of 8207 genes, of which 1686 had FC > 2 and p < 0.05 (20.5%); these were divided into 1229 downregulated and 457 upregulated genes (see the Supplementary Dataset for the complete list of transcripts with their fold-changes and p values).

We also calculated the percentage of DEGs per chromosome for the upregulated and downregulated genes in the three treatments. Interestingly, we found that chromosome 12 had the highest percentage of downregulated genes at temperatures of 24°C and 30°C (the treatments with the highest numbers of DEGs), with values of 21.3% and 28.7%, respectively. In other words, chromosome 12 was the most affected at these temperatures, as shown by the downregulation of a large proportion of its genes. Whereas, the chromosome with the highest proportion of upregulated genes at 30°C (the treatment with the most DEGs) was chromosome 25, with 9.3% of genes upregulated [Supplementary data (Fig. 9)].

After categorising the DEGs into two categories, up- and downregulated genes, we performed an analysis to determine the ontology of these genes. In this way, we found that the three treatments had similar patterns since a large number of genes encode hypothetical proteins, followed by fewer domains, families and proteins of unknown function. Another observed pattern was that the downregulated genes were associated with fewer GO terms than the upregulated genes, even though the number of downregulated genes was greater. Thus, we concluded that the large number of downregulated genes had similar or redundant biological functions, mostly associated with biological processes, organonitrogen compound metabolic processes and protein metabolic processes, among others (Fig. 7D).

In Fig. 7B and 7D, we illustrate the GO terms with the maximum proportion of up- and downregulated genes associated with them for the three assessed temperatures. All of the GO analyses generated a p value < 0.05. In the case of the upregulated genes, we observed a pattern in which, at 24°C, a great proportion of genes were associated with all of the GO terms represented in Fig. 7B, so, the upregulation of a superior number of genes associated with biological, cellular, biosynthetic and metabolic processes at 24°C compared with the number of genes associated with these same GO terms at other temperatures may be the reason why parasites under 24°C were less affected in terms of growth and cellular concentration (Fig. 1).

Despite the large number of DEGs associated with metabolic, biological and cellular processes, a small proportion of other genes were associated with GO terms related to responses to stimuli; for example, some genes that were upregulated at 28°C and 30°C were related to responses to external stimuli. Furthermore, some genes related to transmembrane transport at 30°C were up- or downregulated, with downregulation being predominant.

The differentially expressed genes were unique at each temperature - As we described above, numerous GO terms were shared among the different temperature treatments; this is in part a consequence of the DEGs shared between treatments (Fig. 7A,C). Next, we evaluated the individual DEGs that were unique for each temperature and the DEGs shared between two or three temperatures. We concluded that the DEGs uniquely up- or downregulated following each treatment were expressed as a direct response to the temperature change.

We evaluated the unique DEGs for both up- and downregulated genes in the three treatments, and the 10% of genes with the largest change in their expression were compared with the expression at 26°C (log-fold change). At 24°C, the gene with the largest log-fold change (2.834) encoded a protein kinase (LbrM.34.1040), followed by other genes encoding ribosomal genes (Table I) and a gene encoding an activator of HSP90 ATPase (LbrM.18.0230), which showed an increase in expression with a log FC of 1.138 and a p value = 3.261E-103 (data not shown, see Supplementary Dataset). By contrast, among the genes downregulated at this temperature, the gene with the largest decrease in expression was encoded by an amastin-like surface protein (with a log FC of -2.815), followed by a gene cluster that encodes a tuzin-like protein (LbrM.20.2420) and another amastin-like surface protein (LbrM.20.2410) (Table I).

By contrast, the genes that were upregulated only at 28°C were found to be associated principally with transcriptional and translational processes, as indicated in
Table II. Among the 10% most upregulated genes, the majority were involved in these processes. Whereas among the downregulated genes, across all of the treatments, we also observed the downregulation of a gene that encodes an amastin-like surface protein (LbrM.24.1600) (Table II) and the downregulation of other genes principally associated with metabolic processes.

Similarly, of the 200 genes uniquely overexpressed at 30°C (Fig. 7A), the majority were related to metabolic, biosynthetic, transcriptional and translational processes, as shown in Table III. In this table, the genes with the highest log FC values encoded tRNA-Gly and tRNA-Asn. Although the promastigotes at 30°C exhibited upregulation of four genes directly associated with the stress response generated by the increased temperature, these included two encoding HSPs [heat shock 70-related protein (LbrM.30.2430, log FC = 1.154 and p = 2.40E-24) and HSP DNAJ (LbrM.27.0500, log FC = 1.059 and p = 1.485E-21)] and two encoding DNAJ domain-containing proteins (LbrM.17.0050, log FC = 1.169 and p = 1.049E-20; and LbrM.32.0670, log FC = 1.054 and p = 1.124E-22), data not shown, see Supplementary Dataset. By contrast, among the downregulated genes at 30°C, a gene encoding an ABC transporter (LbrM.27.1050, log FC = -1.35 and p = 5.99E-07) was detected.

Differentially expressed genes shared between temperatures - As well as evaluating the genes expressed uniquely at each temperature, we also evaluated which genes were changed in expression in response to two or all of the test temperatures to determine which genes were up- or downregulated irrespective of the extent of the temperature shift. Among the genes that were altered in expression at all three temperatures, we found downregulated genes that encoded the same products as previously reported genes, such as those encoding (1) amastin-like surface proteins (LbrM.18.0460, LbrM.18.0470, LbrM.13.1330, LbrM.10.1520, LbrM.08.0670, LbrM.08.0680, LbrM.20.0950, LbrM.20.0960, LbrM.20.1080, LbrM.20.4340, LbrM.24.1590, LbrM.35.4370 and LbrM.35.4380), which in this case were overrepresented compared with previous results; (2) ABC transporters (LbrM.02.0350, LbrM.11.1040, LbrM.11.1020 and LbrM.11.0960); and (3) DNAJ domain protein (LbrM.24.1630), also known as HSP40.8)

Among the DEGs shared between the 24°C and 30°C treatments, one gene was upregulated that encodes a stress-inducible protein, STI1 (LbrM.35.0120), with a similar log FC at both temperatures (1.044 and 1.069, respectively, see the Supplementary Dataset). Among the downregulated genes at these same temperatures,
genes encoding ABC transporters (LbrM.06.0010, LbrM.11.1000, LbrM.11.1010 and LbrM.15.0930), an amastin surface glycoprotein (LbrM.28.1210) and heat shock 70-related protein 1 (LbrM.30.2450) were detected. Finally, we evaluated the DEGs shared between the 28°C and 30°C heat treatments and in the majority of cases decreased expression was observed with downregulated genes encoding an amastin-like surface protein, an amastin surface glycoprotein (LbrM.24.1270 and LbrM.27.0650) and ABC transporters (LbrM.15.0820, LbrM.29.0630 and LbrM.29.1750).

**DISCUSSION**

Temperature affected the growth curve of *L. bresilienises*, with 30°C heat treatment having the highest negative effect on parasite concentration (Fig. 1). This may be explained by the general response of promastigotes to higher temperatures as a consequence of host changes, as it has been reported that *in vitro* promastigotes lose their motility, become rounded and express HSPs in response to higher temperatures. Herein, we illustrated the response of the growth curve at lower temperatures and found continuing growth of the parasite. However, the BLP was delayed at 24°C compared with that at 28°C and 30°C. This perhaps reflect the effect of low temperatures on physiological changes in microorganisms and cells such as decreased membrane fluidity, decreased efficiency of transport proteins and a slower rate of growth.

When evaluating changes at the chromosomal level, no variations in ploidy were detected in any of the 35 chromosomes either by analysing allele frequency; moreover, we found no significant variations when evaluating ploidy based on RNA-seq (Fig. 2). In other studies, it has been suggested that ploidy changes are a regular response to environmental change and drug resistance; however, we did not detect any ploidy changes indicating that variations in chromosome copy number may require a longer period of time to be fixed. Future studies should consider whether long-term temperature shifts impact on DNA ploidy. Some chromosomes, particularly chromosome 31, possessed a high number of genes with CNVs following a temperature

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**TABLE I**

Up- and downregulated differentially expressed genes (DEGs) expressed only at 24°C. The selection was based on those genes with the highest 10% of log fold change in their expression compared with the control. The cut-offs were fold change > 2 (log fold change > 1) and p value < 0.05

| Gene ID       | Product description                      | Log2 fold change | p value          |
|---------------|------------------------------------------|------------------|------------------|
| LbrM.34.1040  | Protein kinase, putative                 | 2.834            | 0.048            |
| LbrM.02.0010  | Phosphoglycan beta 1.3 galactosyltransferase 3 | 1.643            | 0.027            |
| LbrM.05.0800  | Methylthioadenosine phosphorylase, putative | 1.541            | 2.907E-34        |
| LbrM.35.3090  | 40S ribosomal protein S24e               | 1.531            | 4.915E-78        |
| LbrM.21.1300  | 40S ribosomal protein S23, putative      | 1.504            | 1.071E-09        |
| LbrM.28.1080  | Ribosomal protein S20, putative         | 1.502            | 6.245E-76        |
| LbrM.28.1100  | Ribosomal protein S20, putative         | 1.474            | 7.291E-14        |
| LbrM.10.1070  | Histone H3                               | 1.465            | 4.3318E-58       |
| LbrM.11.0760  | 40S ribosomal protein S5                 | 1.444            | 3.744E-29        |
| LbrM.35.3980  | 60S ribosomal protein L34, putative     | 1.442            | 8.771E-33        |
| LbrM.31.tRNA1 | tRNA-Ala                                 | 1.431            | 0.002            |
| LbrM.32.2950  | Ribosomal protein L27, putative         | 1.426            | 2.335E-05        |
| LbrM.06.0590  | 60S ribosomal protein L23a, putative    | 1.411            | 4.142E-23        |
| LbrM.34.3760  | 60S ribosomal protein L27/L29, putative | 1.386            | 6.055E-71        |
| LbrM.20.4310  | Amastin-like surface protein, putative  | -2.815           | 1.406E-107       |
| LbrM.20.2420  | Tuzin-like protein                       | -2.056           | 7.607E-20        |
| LbrM.20.2410  | Amastin-like surface protein, putative  | -1.639           | 2.823E-45        |
| LbrM.20.4290  | Amastin-like surface protein, putative  | -1.616           | 1.437E-47        |
| LbrM.18.0060  | Hypothetical protein, conserved         | -1.567           | 1.415E-50        |
| LbrM.28.2110  | Zinc transporter 3, putative            | -1.535           | 5.914E-11        |
| LbrM.32.0330  | Serine/threonine-protein kinase Nek1-related, putative | -1.482 | 3.978E-31 |
| LbrM.12.0160  | Cell division protein kinase, putative  | -1.475           | 2.165E-36        |
| LbrM.18.1000  | Nrap protein, putative                  | -1.468           | 0.001            |
| LbrM.32.3711  | Hypothetical protein, conserved         | -1.466           | 2.869E-23        |
shift compared with the control (Fig. 3). This chromosome is known to be supernumerary in many *Leishmania* species such as *L. major* and *L. peruviana*,(12) and has a copy number of more than two in all sequenced species of *Leishmania*. Hence, this chromosome might be important for *Leishmania* adaptation and further studies are needed to investigate this.

We evaluated the genes with CNVs related with the enriched GO terms associated with every treatment. In first place, Elongation factor 1-alpha genes changes their copy number in the three temperature treatments, it is an important factor in protein transduction because it catalyses the GTP dependent binding of aminoacyl-tRNA to the A-site of ribosomes.(18) We also found for the three treatments CNVs in genes related with cytoskeleton which are fundamental in cell shape, intracellular transport and morphological changes in *Leishmania*. For CNVs changes unique at each temperature, at 24 there was a high read depth in the copy number of genes that encode HSP70 (HSP70 is a chaperone protein that is expressed for environmental adaptation), and a putative transporter-like protein, but at the same time there was an increase in genes coding for ion transport and other membrane transport proteins that has been related with protozoan adaptation.(20) Results from 28°C and 30°C treatments showed CNVs for genes that code ribosome subunits and signalling membrane proteins as well as heat shock proteins which are all important on *Leishmania* adaptation.(6,7)

When the CNVs were evaluated, along with their effects on gene expression, no correlations were found. Therefore, the variations in expression may be due to post-transcriptional regulatory mechanisms such as transsplicing reactions of mRNAs and 3′-polyadenylation,(21) mRNA stability, translational control, and/or mRNA degradation.(22) This conclusion is supported by the fact that at 30°C, the chromosome with the highest proportion of DEGs was chromosome 12, which was the only chromosome that did not show CNVs in its genes (Fig. 3). However, we cannot assume that gene CNVs might be relevant to short-term adaptations to temperature for the slightly variation and the few days of the treatment, this variation could be stochastic so more studies should be done to infer the role that play CNVs in a temperature adaptation.

In addition, we observed a considerable number of DEGs as a possible response to the temperature treatments. One example was the overexpression of HSPs, which were upregulated principally at 30°C but to a lesser extent at 24°C. This may indicate that at more extreme temperatures, the impact of heat stress on the synthesis of HSPs is more noticeable. These upregulated HSPs play important roles in several cellular processes, such as protein folding, assembly, trafficking, activity and degradation.(19) Specifically, the HSPs identified in this study are contained within a complex, since the activator HSP90 ATPase is fundamental for the activation of HSP90,(23) which forms the centre of a chaperone complex known as the HSP90 foldosome. Together with other co-chaperones, such as HSP70, HSP40 and stress-inducible protein 1 (Sti1), HSP90 is implicated in certain functions such as transduction signalling and cell cycle control.(24) In addition, each HSP has other functions, such as, RNA stability and translational control and, in the case of HSP70, in transcript stability.(24) Moreover, these proteins also play other important roles that directly affect the cell cycle, since some of the HSPs, such as HSP90 and several associated chaperones and co-chaperones, are involved in natural modulation pathways that include protein kinases. Examples of this include being substrates for MAP kinase 1, which is crucial for the intracellular survival of *Leishmania*; casein kinase 1.2 catalysing HSP90 phosphorylation to promote promastigote growth,(25) and the interaction between HSP90 and Sti1 to promote the fast-growing insect and mammalian host stages of the parasite.(26)

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**TABLE II**

The 10% of genes with the highest log fold change in their expression compared with the control. Among the up- and downregulated differentially expressed genes (DEGs) only expressed at 28°C. The cut-offs were fold change > 2 (log fold change > 1) and p value < 0.05.

| Gene ID       | Product description                        | Log2 fold change | p value  |
|--------------|--------------------------------------------|------------------|----------|
| LbrM.11.tRNA5| tRNA-Arg                                   | 1.485            | 0.000    |
| LbrM.34.4080 | Splicing factor 3B subunit 10 (SF3b10), putative | 1.428            | 7.672E-36|
| LbrM.13.1240 | Ran-binding protein 1, putative            | 1.338            | 2.527E-13|
| LbrM.32.0060 | Nuclear segregation protein, putative      | 1.261            | 4.836E-12|
| LbrM.01.0350 | Hypothetical protein, conserved            | 1.245            | 3.458E-08|
| LbrM.16.0010 | Anti-silencing protein a-like protein       | 1.224            | 5.478E-16|
| LbrM.04.0420 | Hypothetical protein                       | -3.243           | 0.007    |
| LbrM.23.snRNA2| Small nuclear RNA, U3 snRNA                | -3.052           | 0.002    |
| LbrM.24.1600 | Amastin-like surface protein-like protein   | -2.369           | 0.002    |
| LbrM.31.3640 | Phosphoglycan beta 1.3 galactosyltransferase 5 (fragment) | -1.894         | 0.038    |
| LbrM.08.0700 | SLACS                                      | -1.690           | 2.220E-05|
TABLE III

The up- and downregulated differentially expressed genes (DEGs) with the 10% highest values in their expression compared with the control expressed only at 30°C. The cut-offs were fold change > 2 (log fold change > 1) and p value < 0.05

| Gene ID       | Product description                                             | Log2 fold change | p value       |
|---------------|------------------------------------------------------------------|------------------|---------------|
| LbrM.11.tRNA3 | tRNA-Gly                                                         | 4.669            | 0.003         |
| LbrM.10.tRNA2 | tRNA-Asn                                                         | 2.062            | 0.034         |
| LbrM.02.0490  | Hypothetical protein, conserved                                 | 1.587            | 4.756E-13     |
| LbrM.16.1460  | Trafficking protein particle complex subunit-like protein       | 1.416            | 6.804E-30     |
| LbrM.34.3560  | Methyltransferase domain-containing protein, putative           | 1.396            | 5.593E-83     |
| LbrM.20.0220  | CS domain-containing protein, putative                          | 1.393            | 8.896E-55     |
| LbrM.20.1400  | Hypothetical protein, conserved                                 | 1.393            | 1.831E-27     |
| LbrM.02.0140  | Hypothetical protein, conserved                                 | 1.390            | 2.781E-35     |
| LbrM.34.1830  | Hypothetical protein, unknown function                          | 1.386            | 2.703E-54     |
| LbrM.20.3420  | Ribosomal protein L14, putative                                 | 1.357            | 2.475E-20     |
| LbrM.09.1410  | Hypothetical protein, conserved                                 | 1.353            | 2.370E-28     |
| LbrM.06.0130  | Hypothetical protein, conserved                                 | 1.353            | 5.338E-25     |
| LbrM.30.2970  | Hypothetical protein, conserved                                 | 1.309            | 1.168E-19     |
| LbrM.20.0820  | Serine/threonine-protein phosphatase PP1, putative              | 1.307            | 6.336E-68     |
| LbrM.29.1820  | Histone H2A, putative                                           | 1.301            | 2.6739E-30    |
| LbrM.04.1160  | Hypothetical protein, conserved                                 | 1.297            | 9.194E-36     |
| LbrM.28.0950  | Hypothetical protein, conserved                                 | 1.296            | 8.645E-34     |
| LbrM.27.0170  | SET domain-containing protein, putative                         | 1.291            | 1.269E-34     |
| LbrM.12.0690  | Hypothetical protein, conserved                                 | 1.287            | 3.706E-28     |
| LbrM.34.4350  | Zinc-binding domain-containing protein, putative                | 1.282            | 2.449E-19     |
| LbrM.33.2020  | Macrophage migration inhibitory factor-like protein             | -2.464           | 1.617E-07     |
| LbrM.05.1210  | Surface antigen-like protein                                    | -2.300           | 2.750E-12     |
| LbrM.34.0520  | Proteophosphoglycan ppg3, putative (fragment)                   | -2.218           | 2.398E-19     |
| LbrM.10.0380  | Folate/bioterin transporter, putative                           | -2.146           | 2.157E-13     |
| LbrM.01.0720  | Protein kinase, putative                                        | -2.142           | 4.29E-22      |
| LbrM.31.3030  | Hypothetical protein, conserved                                 | -2.084           | 1.483E-17     |
| LbrM.14.0540  | Hypothetical protein, unknown function                          | -1.987           | 5.258E-07     |
| LbrM.13.0100  | SURF1 family, putative                                          | -1.987           | 1.669E-13     |
| LbrM.01.0260  | Long-chain-fatty-acid-CoA ligase, putative (fragment)            | -1.937           | 2.211E-13     |
| LbrM.30.0340  | Hypothetical protein, conserved                                 | -1.919           | 5.127E-15     |
| LbrM.34.4200  | Hypothetical protein, unknown function                          | -1.906           | 2.559E-24     |
| LbrM.31.1450  | Pyrophosphate-energized vacuolar membrane proton pump 1, putative | -1.870           | 5.926E-16     |
| LbrM.29.2710  | Hypothetical protein, conserved                                 | -1.797           | 3.238E-17     |
| LbrM.20.5790  | TBC1 domain family member 20/GTPase, putative                  | -1.718           | 3.01E-08      |
| LbrM.22.0010  | CLN3 protein, putative                                          | -1.716           | 1.25E-16      |
| LbrM.27.1000  | Protein of unknown function (DUF1295), putative                 | -1.669           | 2.388E-31     |
| LbrM.23.1890  | COG4 transport protein, putative                                | -1.660           | 3.379E-17     |
| LbrM.18.0650  | RNA binding protein, putative                                   | -1.642           | 1.583E-16     |
| LbrM.20.5530  | Small myristoylated protein                                     | -1.630           | 1.489E-13     |
Other genes found to be expressed in association with the three treatments were those encoding the amastin surface-like proteins, which showed a pattern of downregulation under the three temperatures compared with the control. These proteins are members of a multigene family encoding glycoproteins that are important components of the parasite surface, being involved in host-parasite interactions and playing a fundamental role during infection.\(^{(27)}\) Therefore, a reduction in the expression of these genes could affect these processes, as was reported by Cardoso de Paiva et al.,\(^{(27)}\) who described that amastin knockdown generated a reduction in the viability of intracellular amastigotes.\(^{(27)}\)

As reported in the current study, other genes that were underexpressed at all of the test temperatures were those that encode the ABC transporters,\(^{(28)}\) which have been reported to be important in the infection process.\(^{(28,29)}\) The transcriptome profile described above shared similarities with that reported by Rastrojo et al.,\(^{(30)}\) in which \(L.\ major\) promastigotes were exposed to heat shock at 37\(^\circ\)C for two hours. They reported upregulation of genes that encode for several HSPs, which was consistent with our results; in addition, the most downregulated transcript in their study was an ABC transporter, which was also detected in our study, and the amastin-encoding genes were also found to be downregulated in both studies.\(^{(30)}\) These results confirmed that these genes are sensitive to temperature shifts.

In conclusion, our study provides evidence that \(L.\ braziliensis\) promastigotes exhibit characteristic changes in DNA and mRNA response to short-term temperature stress \textit{in vitro}. This evidence included changes in the growth curves, CNVs in 3\% of the genes evaluated at each temperature, up- or downregulation of a range of genes associated with temperature stress (HSPs were upregulated at 30\(^\circ\)C, and amastin-like proteins and ABC transporters were downregulated at the three temperatures) and variations in expression of many genes associated with the GO terms of cellular, biosynthetic and biological processes. These findings revealed the great impact of temperature on the transcriptome profile, leading to rapid and efficient cellular responses. Furthermore, we found that temperature has a negative effect on the growth curves of \(L.\ braziliensis\), at least in the short-term. Our findings provide the first insights into the genomic and transcriptomic changes in \(L.\ braziliensis\) following temperature shifts, confirming the important role played by this abiotic factor in biological processes in the parasite over the short-term. One limitation of this study was the use of promastigotes under controlled conditions. Future studies should consider the impact of temperature shifts on promastigotes infecting sandflies and on amastigotes in the mammalian host to determine the holistic factors impacting the dynamics of the genome and transcriptome of \(Leishmania\) parasites.

The data set generated during the current study was deposited at DDBJ/ENA/GenBank under the accession number PRJEB31852 (ERP114463).
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AUTHORS’ CONTRIBUTION

NB and NV performed all experiments, analysed the data, wrote the manuscript, and prepared all figures and tables; LC contributed to the experiments, generation of the figures and revision of the manuscript; LP collaborated with the RNA and DNA data analysis and revised the manuscript; JR designed and supervised the project, revised the manuscript, and was responsible for the funding. All the authors read and approved the final version of the manuscript. The authors declare that there are no conflicts of interest.

REFERENCES

1. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012; 7(5): e35671. https://doi.org/10.1371/journal.pone.0035671.

2. WHO - World Health Organization. Leishmaniasis: epidemiological situation. 2019. Available from: https://www.who.int/leishmaniasis/burden/en/.

3. Hlavacova J, Votycka J, Volf P. The effect of temperature on Leishmania (Kinetoplastida: Trypanosomatidae) development in sand flies. J Med Entomol. 2013; 50(5): 955-8. https://doi.org/10.1603/ME10353.

4. Zilberstein D, Shapiro M. The role of pH and temperature in the development of Leishmania parasites. Ann Rev Microbiol. 1994; 48(1): 449-70.

5. Koch LK, Kochmann J, Klimpel S, Cunze S. Modeling the climatic suitability of leishmaniasis vector species in Europe. Scie Rep. 2017; 7(1): 1-10. https://doi.org/10.1038/s41598-017-13822-1.

6. Lawrence F, Robert-Gero M. Induction of heat shock and stress proteins promastigotes of three Leishmania species. PNAS USA. 1985; 82(13): 4414-7.

7. Toye P, Remold H. The influence of temperature and serum deprivation on the synthesis of heat-shock proteins and alpha and beta tubulin in promastigotes of Leishmania major. Mol Biochem Parasitol. 1989; 35(1): 1-10.

8. Folgueira C, Quijada L, Soto M, Abanades DR, Alonso C, Requena JM. The translational efficiencies of the two Leishmania in-factum HSP70 mRNAs, differing in their 3′-untranslated regions, are affected by shifts in the temperature of growth through different mechanisms. J Biol Chem. 2005; 280(42): 35172-83. https://doi.org/10.1074/jbc.M505559200.

9. Downing T, Imamura H, Decuyper S, Clark TG, Coombs GH, Cotton JA, et al. Whole genome sequencing of multiple Leishmania donovani clinical isolates provides insights into population structure and mechanisms of drug resistance. Genome Res. 2011; 21(12): 2143-56. https://doi.org/10.1101/gr.123430.111.Freely.

10. Rastrojo A, Garcia-Hernández R, Vargas P, Camacho E, Corvo L, Imamura H, et al. Genomic and transcriptomic alterations in Leishmania donovani lines experimentally resistant to antileishmanial drugs. Int J Parasitol Drugs Drug Resist. 2018; 8(2): 246-64. https://doi.org/10.1016/j.ijpddr.2018.04.002.

11. Bussotti G, Gouzelou E, Boitè MC, Kherachi I, Harrat Z, Eddaiakra N, et al. Leishmania genome dynamics during environmental adaptation reveal strain-specific differences in gene copy number variation, karyotype instability, and telomeric amplification. mBio. 2018; 9(6): pii: e01399-18. https://doi.org/10.1128/mBio.01399-18.

12. Valdivia HO, Reis-Cunha JL, Rodrigues-Luiz GF, Baptista RP, Baldevani GC, Gerbasi RV, et al. Comparative genomic analysis of Leishmania (Viannia) peruviana and Leishmania (Viannia) braziliensis. BMC Genomics. 2015; 16(715). https://doi.org/10.1186/s12864-015-1928-z.

13. Dumetz F, Imamura H, Sanders M, Seblov V, Mysova J, Pescher P. Modulation of aneuploidy in Leishmania in vitro and in vivo environments and its impact on gene expression. mBio. 2017; 8(3): 1-14. https://doi.org/10.1128/mBio.00599-17.

14. Ianitorno SA, Durrant C, Khan A, Sanders MJ, Beverley SM, Warren WC, et al. Gene expression in Leishmania is regulated predominantly by gene dosage. mBio. 2017; 8(5): e01393-17. https://doi.org/10.1128/mBio.01393-17.

15. Rogers MB, Hilley JD, Dickens NJ, Wilkes J, Bates PA, Depledge DP, et al. Chromosome and gene copy number variation allow major structural change between species and strains of Leishmania. Genome Res. 2011; 21(12): 2129-42. https://doi.org/10.1101/gr.122945.111.

16. Nedwell DB. Effect of low temperature on microbial growth: lowered affinity for substrates limits growth at low temperature. FEMS Microbiol Ecol. 1999; 30(2): 101-11. https://doi.org/10.1016/0168-6496(97)00048-9.

17. Mannaert A, Downing T, Imamura H, Dujardin JC. Adaptive mechanisms in pathogens: universal aneuploidy in Leishmania. Trends in Parasitol. 2012; 28(9): 370-6. https://doi.org/10.1016/j.pt.2012.06.003.

18. Lopez M, Cherkasov A, Nandan D. Molecular architecture of Leishmania EF-la reveals a novel site that may modulate protein translation: a possible target for drug development. Biochem Biophys Res Commun. 2007; 356(4): 886-92. https://doi.org/10.1016/j.bbrc.2007.03.077.

19. Ramirez CA, Requena JM, Puerta CJ. Alpha tubulin genes from Leishmania braziliensis: genomic organization, gene structure and insights on their expression. BMC Genomics. 2013; 14(454). https://doi.org/10.1186/1471-2164-14-454.

20. Bakker-Grunwald T. Ion transport in parasitic protozoa. J Exp Biol. 1992; 172: 311-12.

21. Kramer S. Developmental regulation of gene expression in the absence of transcriptional control: the case of kinetoplastids. Mol Biochem Parasitol. 2012; 181(2): 61-72. https://doi.org/10.1016/j.molbiopara.2011.10.002.

22. Späth GF, Drini S, Rachidi N. A touch of Zen: post-translation al regulation of the Leishmania stress response. Cell. 2015; 16(5): 632-8. https://doi.org/10.1016/j.cell.2015.04.015.

23. Zilha A, Garlapati S, Dahan E, Yaolsky V, Shapira M. Developmental regulation of heat shock protein 83 in Leishmania. J Biol Chem. 2001; 276(51): 47922-9. https://doi.org/10.1074/jbc.M102712200.

24. Seraphim TV, Alves MM, Silva IM, Gomes FER, Silva KP, Murta SMF, et al. Low resolution structural studies indicate that the activator of Hsp90 ATPase 1 (Ah1) of Leishmania braziliensis has an elongated shape which allows its interaction with both N- and M-domains of Hsp90. PLoS ONE. 2013; 8(6): e66822. https://doi.org/10.1371/journal.pone.0066822.

25. Bifeld E, Lorenzen S, Bartsch K, Vasquez J-J, Siegel T, Clos J. Ribosome profiling reveals HSP90 inhibitor effects on stage-specific protein synthesis in Leishmania donovani. mSystems. 2018; 3(6): e00214-18. https://doi.org/10.1128/mSystems.00214-18.

26. Hombach A, Ommen G, Chrobak M, Clos J. The Hsp90â–Sti1 interaction is critical for Leishmania donovani proliferation in both life cycle stages. Cell Microbiol. 2013; 15(4): 585-600. https://doi.org/10.1111/cmi.12057.
27. Paiva RMC, Grazielle-Silva V, Cardoso MS, Nakagaki BN, Mendonça-Neto RP, Canavaci AM, et al. Amastin knockdown in *Leishmania braziliensis* affects parasite-macrophage interaction and results in impaired viability of intracellular amastigotes. PLoS Pathogens. 2015; 11(12): 1-24. https://doi.org/10.1371/journal.ppat.1005296.

28. Leprohon P, Le D, Girard I, Papadopoulou B, Ouellette M. Modulation of *Leishmania* ABC protein gene expression through life stages and among drug-resistant parasites. Eukaryotic Cell. 2006; 5(10): 1713-25. https://doi.org/10.1128/EC.00152-06.

29. Parodi-Talice A, Araújo IM, Torres C, Pérez-Victoria JM, Gamarro F, Castany S. The overexpression of a new ABC transporter in *Leishmania* is related to phospholipid trafficking and reduced infectivity. Biochim Biophys Acta. 2003; 1612(2): 195-207. https://doi.org/10.1016/S0005-2736(03)00131-7.

30. Rastrojo A, Corvo L, Lombraña R, Solana JC, Aguado B, Requejo JM. Analysis by RNA-seq of transcriptomic changes elicited by heat shock in *Leishmania major*. Sci Rep. 2019; 9(6919): 1-18. https://doi.org/10.1038/s41598-019-43354-9.