Transcriptome profiles evaluation of *Leishmania braziliensis* promastigotes subjected to temperature shifts *in vitro*

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Abstract

The increasing of the temperature is one of the principal consequences of the climate change, which affect human populations due of the emergence and re-emergency of infection diseases. The Leishmaniases are diseases cause by protozoans' parasites of the genus *Leishmania*; these diseases are composed by different clinical manifestations, one of the most important in the New World is Cutaneous Leishmaniasis for which the most common causative species is *Leishmania braziliensis*. This species as the other members of the Trypanosomatidae family present a genomic plasticity and a particular gene expression regulation that allow to the parasites to adapt and response to several stimulus, for that reason the aim of this study is evaluate the transcriptome profiles of *L. braziliensis* promastigotes subjected to temperature shifts. To reach this aim the authors performed an RNA-Seq that permitted to find several genes associated with a direct response to the treatments; also, through the growth curves done the authors evidenced a decrease in the cell proliferation in all the temperatures tested, where the most affected was 30°C. The results obtained in this study demonstrated a fast response of *L. braziliensis* promastigotes to temperature shifts.

Key words

Transcriptomic, heat stress, response to external stimulus, growth curves.

Palabras claves

Transcriptómica, estrés térmico, respuesta a estímulos externos, curvas de crecimiento.

Introduction

In recent decades, there has been a drastic increase of the global temperature as part of climate change (Liang & Gong, 2017), affecting human populations due to the associated scarcity of resources and the emergence and re-emergence of infectious diseases. Several tropical pathologies are relevant in this context, such as zika, chikungunya, malaria and leishmaniases (Lafferty & Mordecai, 2016; Wu et al., 2016). The leishmaniases are caused by a protozoan belonging to the genus *Leishmania* (Teixeira et al., 2013) which feature a variety of clinical manifestations: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML) and visceral leishmaniasis (LV). 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 (Ramírez et al., 2016). 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 countries such as Afghanistan, Algeria, Brazil, Iran, Syria and Colombia. CL is also the most important and common clinical manifestation in the New World; it is characterized by ulcerative and deformative lesions, for which the most common causative species is *Leishmania braziliensis* (Luz H. Patino et al., 2017; Ramírez et al., 2016).

Temperature is a factor that determines whether this parasite can develop appropriately, as was reported in a study by Hlavacova *et al*. They evaluated the effect of temperature on the life cycle of three *Leishmania* species inside two different species of vector (sandflies); they concluded that the response to temperature is species-specific (Hlavacova, et al., 2013). Other researchers also evaluated the effects of this environmental factor on *Leishmania* in view of the fact that these parasites already encounter temperature shifts during their life cycle (in the sand fly the temperature ranges from 22 to 28°C, while in the mammalian host it ranges from 31 to 37°C) (Leon, et al., 1995; Zilberstein & Shapira, 1994). These studies reported different results depending on the temperature treatment and the *Leishmania* species, some of the results obtained were an increase in the parasites multiply, morphological changes (the parasites acquired a round shape), loss of motility (Zilberstein & Shapira, 1994), changes in parasites differentiation rate and in their surviving (Leon et al., 1995). However, there have been few studies aimed at understanding the biology of *Leishmania* when subjected to an increase of temperature and as far as we know, not one when subjected to a decrease of this variable. Most studies have focused on the impact of temperature shifts on the distribution of the vectors and the association of this with the number of leishmaniasis cases (Cardenas, et al., 2007; González et al., 2010; Koch, et al., 2017; Rajesh & Sanjay, 2013). Other studies have evaluated this variable on promastigotes of *L. infantum*, *L. major*,

L. tropica, *L. enrietti* and *L. donovani* 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 of temperature (Folgueira et al., 2005; Lawrence & Robertgero, 1985; Toye & Remold, 1989). Nonetheless, to the best of our knowledge, no study has particularly focused on the effect of temperature on *Leishmania*, including on its transcriptome.

Even though, other studies on *Leishmania* let understand how the parasite biology change in response to other stress situations which could be similar under temperature stress. Rastrojo *et al.* found that somy changes, amplifications and deletions are the mechanisms for which the parasite acquires the resistance to antileishmanial drugs (Rastrojo et al., 2018). Dumetz *et al.*, determined the impact of host change in *L. donovani* where the aneuploidy was higher in promastigotes *in vitro* than in amastigotes *in vivo* (Dumetz et al., 2017). Other important finding of this study is that some chromosomal copy number variation had a correlation with its transcriptomic profile but also there were a transcriptome response independent to the aneuploidy (Dumetz et al., 2017). Bussotti *et al.*, reveal in *L. major*, *L. tropica*, and *L. infantum* two ways of environment adaptation at short term (culture passaging), changes in aneuploidy and telomeric amplification (Bussotti et al., 2018), which could play important roles in the adaptation since the chromosomal amplification might be under selection (Barja et al., 2017; Bussotti et al., 2018). At long term adaptation there was a strain specific copy number variation of single genes that may alleviate the necessity of chromosome duplication (Bussotti et al., 2018). There are also other studies in *Leishmania* that show the importance of genome and transcriptome changes not only in the response to drugs like trivalent antimony, amphotericin, miltesosine and paramomycin (Downing, et al., 2011; Mondelaers et al., 2016; Rastrojo et al., 2018; Shaw et al., 2016; Vanaerschot et al., 2012) but also in *Leishmania* variation between strains and species where there are more differences in gene copy number and aneuploidy instead of Single-Nucleotide Polymorphisms (SNP) (Coughlan et al., 2018; Dujardin, et al., 2014; Valdivia et al., 2015); which is why the genotypic plasticity of *Leishmania* plays an important role in the adaptation of some stressful conditions. Although, these kinds of comparisons have not been taken deeply in *L. braziliensis*.

L. braziliensis as the other members of the family Trypanosomatidae present a gene organization in polycistronic groups and an absence of genetic regulation by promoters of the RNA polymerase II, for that reason the principal mechanisms of gene expression regulation is post-transcriptional, which include mRNA stability, mRNA degradation, and a possible regulation due to gene dosage

(Iantorno et al., 2017), as was reported by Iantorno, *et al*. who found that the 85% of differences of the gene expression in *Leishmania tropica*'s isolations is explain by copy number variations at chromosomal and gene level (Iantorno et al., 2017). Due to the high genomic plasticity and the particular gene expression regulation in this family of parasites, the next generation sequencing has been increased the knowledge of the trypanosomatids biology, since technologies as transcriptomics have allowed to reveal important molecular changes that permit understand how differential expression generate a response to different external stimulus (Luz Helena Patino & Ramírez, 2017).

Taking account that the increase of the temperature during the XXI century is approximately from 1 to 5.8°C (Lean & Rind, 2009; Wu et al., 2016) as a consequence of the climate change, we used the next generation sequencing (RNA-Seq) to determine the possible transcriptome impact that could have *L. braziliensis* promastigotes to shifts of the temperature giving an insight of how the parasite behavior would be in a future climate change scenario. Our study shows for the first time the biological response at transcriptomic level of *L. braziliensis* promastigotes cultivated *in vitro* and subjected to temperature shifts, being the first approach not only for this species but also for the genus, in addition, we reported the effect when submitted to a lower temperature, a perspective which have not been studied until the moment.

Materials and methods

Culture conditions and growth curves

Promastigotes of *Leishmania braziliensis* from the strain MHOM/BR75/M2904 were cultivated in the growth medium Roswell Park Memorial Institute (RPMI) (Sigma-Aldrich) supplemented with 10% heat-inactivated foetal bovine serum (Invitrogen). The parasites were incubated at four different temperatures, which were chosen in consideration of the predicted temperature increase during the current century (1–5.8°C); thus, the temperatures assessed were 24, 26, 28 and 30°C. Here, 26°C was used as a control temperature because it is a standard temperature *in vitro* culture. We prepared cultures with three replicates per temperature in 25 cm^3 plates with an initial concentration of 1×10^6 parasites/mL in each. The parasite density for all culture replicates at each temperature was quantified using a Neubauer chamber for 7 consecutive days. This quantification was used to create 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 analyzed normality using the Kolmogorov–Smirnov test and subsequently used a Dunnett test of multiple comparisons to determine the day on which the logarithmic phase started. To determinate if there was a difference between the evaluated temperatures, we did a two-way ANOVA test. The p values < 0.05 were considered like statistically significant.

Isolation of RNA

RNA extraction was performed on the day when the logarithmic phase began, as defined by the parasite growth curves at each temperature. Total RNA was extracted from two biological independent replicates, using the RNeasy Plus Mini Kit (QIAGEN) following the manufacturer's instructions, to control the sequencing process two technical replicates from each biological replicate were including. The concentration and quality of the RNA samples were quantified using NanoDrop 2000 (Thermo Scientific™), and the integrity was assessed by electrophoresis in a 1% agarose gel. All samples had A_{260}/A_{280} ratios higher than 2.0.

Transcriptome sequencing

RNA was obtained from cultures of the strain of *Leishmania braziliensis* known as MHOM/BR75/M2904. Once we extracted the RNA, it was sent to be sequenced by Illumina HiSeq X-TEN. The libraries prepared were Strand-specific TrueSeq RNA-seq Library Prep (Illumina) with insert size of 350 bp. The reads had a size of 2 × 150 bp. The sequencing was performed by Novogene Bioinformatics Technology Co., Ltd, Beijing, China. The software FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was conducted to determine the reliability of the sequencing.

RNA read mapping

The reads were mapped to a genome reference, *L. braziliensis* MHOM BR75 M2904, through the software SMALT v0.7.4 (www.sanger.ac.uk/resources/software/smalt/), and using 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/).

Data analysis

The levels of transcripts were quantified by assessing read depth, as described previously (Downing et al., 2011; Imamura et al., 2016). The relative RNA-based somy (RNA-S) per chromosome was computed using the average read depth of transcripts and heatmaps were created using the R package Heatmap3. The somy range is the same as described previously in the DNA analysis section and based on the study by Rogers *et al*. (Rogers et al., 2011). The obtained results were statistically analyzed through two-way ANOVA (software GraphPad Prism) to determine whether the treatments were associated with a significant change of RNA-S compared with the results at 26°C. To assess the impact of the gene CNV 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 p value < 0.05 to define DEGs. The proportion of differentially expressed genes per chromosome is defined as follows: (number of DEGs per chromosome) / (number of total genes per chromosome) × 100. 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. The Venn diagrams and GO figures were performed using Microsoft Office tools.

Results

Growth curves of *L. braziliensis* promastigotes incubated *in vitro* under different temperatures

We determined the growth curves and the BLP for each temperature treatment through a daily count of the parasites, which were maintained under the same conditions. At the control temperature, promastigotes reached the BLP on the sixth day, and we did not see a decrease of the cell density in the control cultures (26°C) during the days assessed (Figure 1). This latter behavior was also seen in promastigotes incubated at 24°C, which did not exhibit a decrease of cell density during the experiment, but under this temperature the parasites changed the day on which the BLP was reached (third day of the experiment). We also observed that the number of parasites after the third day increased only slightly and slowly during the rest of the experiment (Figure 1). In contrast, the growth curves of the promastigotes at temperatures of 28 and 30°C were similar; in both cases, the BLP was on the second day, followed by a decrease of the cell density on the last days of the

experiment (Figure 1). Despite this similarity in pattern between these two highest temperatures, the magnitude of the cell density differed between them, with there being fewer parasites at 30°C (Figure 1). Notably, the growth curves were significantly different among each temperature (*p* value < 0.05).

Figure 1. Effect of temperature on the growth curve of *L. braziliensis* promastigotes. Parasites were cultured at four different temperatures: 26°C (orange), as the control temperature; 24°C (light blue); 28°C (light green); and 30°C (yellow). The data of beginning of the logarithmic phase (BLP) and the *p* value for the comparison between the growth curves is mention. The parasite growth was monitored daily for 7 days through a Neubauer chamber. Bars represent standard errors obtained from three independent experiments. The cultures had an initial concentration of 1×10^6 parasites/mL. The days of quantification are represented on the x-axis and the concentration of parasites per millimeter is express on the y-axis.

Somy value based on RNA sequencing

For each of the 35 chromosomes, we computed the mean transcript level to determine the somy value based on the results of RNA-Seq (RNA-S). Then, we compared this RNA-S with the results obtained of the somy value based on a DNA-seq (Vásquez, in preparation), the author did not observe a difference in aneuploidy between the control and the treatments in any of the chromosomes. We also observed no difference in aneuploidy based on RNA-S (Figure 2), for each comparison between the control and each treatment we obtained the following results: between 26 and 24°C, the *p* value in two-way ANOVA was 0.073, between 26 and 28°C it was 0.220, and between 26 and 30°C it was 0.144. We expected this result since variations in somy values (DNA) are generally mirrored by alterations in RNA-S (Dumetz et al., 2017). Thus, as Vásquez did not observe variations in DNA-based somy (in preparation), we also observed no significant variations in RNA-S (Figure 2).

Figure 2. RNA-based somy values. Heatmaps show the aneuploidy of each of the 35 chromosomes calculated through the results obtained by high-throughput RNA sequencing. The color keys

indicates the somy value (S), which ranges from 1 to 5, where monosomy is when $S < 1.5$, disomy $1.5 \leq S < 2.5$, trisomy $2.5 \leq S < 3.5$, tetrasomy $3.5 \leq S < 4.5$ and pentasomy $4.5 \leq S < 5$, as previously described (Rogers et al., 2011). The heatmap includes the four replicates (two biological and two technical) indicated between the letters "Lb" (abbreviation for *L. braziliensis*) and the last number (the temperature assessed); for instance, Lb_1_30 is the first replicate at the temperature of 30°C.

Evaluation of copy number variation (CNV) at the gene level and the impact in the transcriptome profiles

Based in the results of the gene CNVs obtained in the study performed by Vásquez, where the author evaluated the impact that could have the temperature shifts in the genome of *L. braziliensis* promastigotes (Vásquez, in preparation), we evaluated their impact at the transcriptome level. First, we wanted to establish whether there was a correlation between the increase/decrease in copy number and the increase/decrease in expression of those genes. For this purpose, we calculated Spearman's correlation coefficient, but the results indicated no correlation between the CNVs and the expression in all treatments, since the r values were near zero and the p values > 0.05. One example of this lack of correlation, is the chromosome 14, in which all the significative genes are with increase copy number variation for the temperatures 28°C and 24°C compared to the control temperature (Vásquez, in preparation), but some of these genes like Lb14.1110 and Lb14.1310 had a decrease in the RNA production compared to 26°C. Despite this, we decided to examine 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. In this way, at 24°C, we found that 15 and 13 genes presented this behavior, respectively, while at 28°C the numbers were 9 and 14, and at 30°C they were 11 and 21.

Interestingly, we found a gene that encode an elongation factor (LbrM.17.0090) that increased its copy number and its expression under the three temperatures. Furthermore, we found other genes that encode to one hypothetical protein (LbrM.04.0230) and an alpha-tubulin (LbrM.29.2700)that increased their copy number and expression at 24 and 28°C. In contrast, some genes decreased their copy number and their expression under the three treatments: two encode to amastin-like surface proteins (LbrM.18.0460 and LbrM.180470), which are also tandem genes, and one encode to NADH-dependent fumarate reductase gene (LbrM.34.1110). At 24 and 30°C, another gene that

encode to NADH-dependent fumarate reductase (LbrM.34.1100) exhibited this decreasing behavior; at 28 and 30°C, genes that encode amastin-like surface protein (LbrM.24.1590) and a beta-tubulin (LbrM.33.0990) also showed these decreases; and at 24 and 28°C, only one gene that encode to poly-zinc finger protein (LbrM.35.1790) shared this behavior. Here, it is worth noting that, at 30°C, there were two genes of particular interest that could have changed expression as a direct response to the temperature treatment. One of these 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) of promastigotes at each assessed temperature

We evaluated the changes in the transcription profiles in response to the different temperatures at which *L. braziliensis* promastigotes were incubated. We compared the gene expression with the results previously obtained at the control temperature (26°C), obtaining values of log fold change. The cut-off chosen was fold change (FC) > 2 (log fold change > 1), as indicated by the dotted line in Figure 3. At the three temperatures, similar patterns of DEGs were identified, with all of them including more downregulated genes than upregulated ones (Figure 3). The promastigotes incubated at 28°C had 920 genes with FC > 2 and p value < 0.05 (11.2%) from a total of 8204 DEGs; of these 920 genes, 639 were downregulated and 281 were upregulated. In a second place, we found the results obtained at 24°C, where 1064 genes had FC > 2 and p value < 0.05 (13%), from a total of 8205 DEGs; of these 1064 genes, 784 were downregulated and 280 upregulated. Finally, in order of lowest to highest number of DEGs, at 30°C the promastigotes showed change in the expression of 8207 genes, of which 1686 had FC > 2 and p value < 0.05 (20.5%); these were divided into 1229 downregulated and 457 upregulated genes.

Figure 3. MA plots demonstrating the differentially expressed genes of *L. braziliensis* promastigotes under each treatment. DEGs (A) at 24°C, (B), 28°C and (C) 30°C. The dotted black lines represent the cut-off of the fold change (log fold change > 1 and < −1).

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 the temperatures of 24 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, since this chromosome had a greater proportion of DEGs than the other 34 chromosomes. After categorizing the DEGs into two categories, up- and downregulated genes, we performed an analysis to determine the ontology of these genes using the database tritrypdb.org, choosing the option biological process to determine the gene function and the major processes in which they are involved. In this way, we found that the three treatments had similar patterns since a large number of genes encode hypothetical proteins were found, followed by fewer genes that encode proteins domains, families and single proteins with 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 could conclude that the large number of downregulated genes have similar or redundant biological functions, mostly associated with biological processes, organonitrogen compound metabolic processes and protein metabolic processes, among others (Figure 4).

Figure 4. Venn diagrams and gene ontology terms of the DEG. Illustrations of the amount of DEG (A) up regulated and (C) down regulated under the three treatments, where the numbers in brackets are the total amount of DEG in each temperature, and representation of the top 10 GO terms with the highest proportion of genes associated with them and the comparison between treatments. GO terms of genes (B) up regulated and (D) down regulated. The X axis is the percentage of genes associated with each GO term calculated taking in count the total amount of genes of each classification (up and down regulated genes). Each color represents a temperature, 24°C (light blue), 28°C (light green), and 30°C (yellow).

In Figure 4, we illustrate the GO terms with the maximum proportion of genes associated with them in both situations, up- and downregulated, in the three assessed temperatures. In the case of the upregulated genes, we observed a pattern in which, at 24°C, the majority of GO terms had the highest proportion of genes associated with them (biological process, cellular process and metabolic processes). The upregulated genes at 28°C showed the second major proportion of genes

associated with these GO terms (Figure 4B). The two patterns described above could be explained by the results obtained experimentally and presented in Figure 1, since *L. braziliensis* promastigotes at 24 and 28°C were less affected in terms of their cell density; for this reason, among the identified genes, there should be a large proportion that are fundamental for growth and appropriate development.

In contrast, the downregulated genes did not show a pattern as mention above, but we could observe that some genes associated with certain metabolic processes were only downregulated at 30°C (Figure 4D). Interestingly, some genes that were upregulated at this temperature were associated with these same GO terms, almost in the same proportion (Figure 4B); thus, while some genes were upregulated, other were downregulated for the same GO term.

Despite the large number of DEGs associated with metabolic, biological and cellular processes, other genes, at small proportions, were associated with GO terms related to responses to stimulus; specifically, some genes that were upregulated at 28 and 30°C were related to responses to external stimulus. Another interesting observation was that some genes that were downregulated at 30°C were related to responses to drugs. Furthermore, some genes related to transmembrane transport at 30°C were up- and downregulated, with a major proportion of genes being in the second category here.

Differentially expressed genes unique in 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 (Figure 4A and 4C). For this reason, we decided to evaluate separately the DEGs that were unique for each temperature and the DEGs shared between two or three temperatures (Figure 4B and 4D). We also concluded that the DEGs uniquely up- and downregulated in 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 we chose the 10% of genes with the largest change in their expression compared with the expression at 26°C (log fold change). At 24°C, the gene with the largest log fold change (2.834)

encode a protein kinase (LbrM.34.1040), followed by other genes with molecular functions such as ribosomal genes (Table 1) and one that encode activator of Hsp90 ATPase (LbrM.18.0230), which showed an increase in expression with a logFC of 1.138 (data not shown). By contrast, among the genes downregulated at this temperature, we found that the gene with the largest decrease in expression encode an amastin-like surface protein (with a logFC−2.815), followed by a gene cluster that encode a tuzin-like protein (LbrM.20.2420) and another amastin-like surface protein (LbrM.20.2410) (Table 1).

Table 1. Up- and downregulated 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 L27A/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

In contrast, within the genes that were upregulated only at 28°C, we found genes associated principally with transcriptional and translational processes, as indicated in Table 2. In this table, the majority of the 10% most upregulated genes are involved in these processes. In the case of the downregulated genes, as we describe above and in common among the treatments, we also observed the downregulation of a gene that encode an amastin-like surface protein (LbrM.24.1600) (Table 2) and the downregulation of other genes principally associated with metabolic processes.

Table 2. The 10% of genes with the highest log fold change in their expression compared with the control. Among the up- and downregulated 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

In a similar way, of the 200 genes uniquely overexpressed at 30°C (Figure 7A), the majority are related to metabolic, biosynthetic, transcript and translational processes, as shown in Table 3. In this table, the genes with the highest log FC values encode tRNA-Gly and tRNA-Asn. Although the promastigotes at 30°C exhibited the upregulation of four genes directly associated with the stress stimulus generated by the increased temperature, two encode heat shock proteins [heat shock 70-related protein (LbrM.30.2430) and heat shock protein DNAJ (LbrM.27.0500)] and two encode DNAJ domain-containing proteins (LbrM.17.0050 and LbrM.32.0670).

Table 3. The up- and downregulated 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.27.0490	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/biopterin 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	3.011E−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	−1.630	1.489E−13
LbrM.34.2530	Hypothetical protein, unknown function	−1.616	5.257E−14
LbrM.04.0790	Hypothetical protein, conserved (fragment)	−1.602	3.788E−14
LbrM.20.5760	Hypothetical protein, conserved	−1.595	2.345E−18
LbrM.35.3160	Phosphatidylinositol 3- and 4-kinase, putative	−1.587	2.433E−65
LbrM.24.0470	Protein of unknown function (DUF3184), putative (fragment)	−1.584	6.276E−37
LbrM.33.2090	Dual-specificity protein kinase, putative	−1.582	6.528E−16
LbrM.05.1110	DNA-directed RNA polymerase I largest subunit	−1.576	2.091E−07
LbrM.08.0450	Hypothetical protein, conserved	−1.570	5.701E−61
LbrM.05.0640	Hypothetical protein, conserved	−1.568	2.325E−11
LbrM.34.1560	Hypothetical protein, conserved	−1.549	2.463E−20
LbrM.03.0030	Hypothetical protein	−1.547	7.055E−17
LbrM.31.0510	Calpain-like protein 2	−1.543	1.024E−22
LbrM.34.2640	Galactokinase-like protein	−1.541	2.970E−31
LbrM.16.1480	Paraflagellar rod protein 2C	−1.538	4.496E−20
LbrM.03.0300	Hypothetical protein	−1.535	1.260E−39
LbrM.05.1130	Hypothetical protein, conserved	−1.531	2.306E−07
LbrM.05.1170	Hypothetical protein, conserved	−1.528	1.149E−22
LbrM.03.0770	Hypothetical protein, conserved	−1.526	4.082E−25
LbrM.12.0350	Myotubularin-related protein, putative	−1.523	2.390E−40

In contrast with the results mentioned previously, at 30°C, there were numerous downregulated genes associated with the temperature increase. Specifically, these were associated with response to and detection of stimulus (GO terms GO: 0050896 and 0051606, respectively), response to chemical (GO: 0042221), response to and detection of abiotic stimulus (GO: 0009628 and 0009582), response to temperature stimulus (GO: 0009266), response to drug (GO: 0042493) and drug transport (GO: 0015893). In addition, there were two genes, encoding sodium stibogluconate resistance proteins (LbrM.31.1110 and LbrM.31.1130), which were not classified under any GO term, but we could infer that they were associated with response to drugs, since sodium

stibogluconate is a medication used to treat leishmaniasis. Notably, some genes were associated with two or more of the GO terms mentioned above; for instance, aquaglyceroporin 1 (LbrM.31.0020) was associated with response to and detection of stimulus, and detection of abiotic stimulus. Finally, one gene that encode an ABC transporter (LbrM.27.1050) was differentially expressed at 30°C showing a downregulation in this treatment.

Differentially expressed genes shared between temperatures

Apart from the evaluation of the genes expressed uniquely at each temperature, we decided to evaluate the genes that had a change in the expression in two or all of the tested temperatures to determine which genes were up- and downregulated irrespective of the extent of the shift of temperature. First, we evaluated the genes differentially expressed at the three temperatures; in the case of the upregulated genes, we found some related to responses to different stimulus and to drug metabolic process (Table S1). We also noted that there were upregulated genes related to response to nutrient levels and to starvation, which could be explained by the change of cell proliferation evidenced in the experiments at the three temperatures (Figure 1); namely, in all cases, there was a decrease in cell density and thus a modification in the growth curves, where the most affected were the promastigotes incubated at 30°C, followed by those at 28°C and finally those at 24°C. This explains why the logFC value was largest at 30°C (Table S1). In the case of the downregulated genes shared among all temperatures, there were genes associated with response to chemical, regulation of cell growth, regulation of cellular response to stress, translation initiation response to stress and translation response to stress (Table S2). We also found other genes previously reported in earlier results, 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) and DNAJ domain protein (LbrM.24.1630), also known as Hsp40 (Folgueira et al., 2005).

Second, we evaluated the DEGs at 24 and 28°C, among which only six genes were up regulated (Figure 4A). Three of these encoded hypothetical proteins, one [tricarboxylate carrier (LbrM.01.0600)] was associated with three GO terms: cation transport (GO: 0006812), ion

transport (GO: 0006811) and transmembrane transport (GO: 0055085), and the other two were not related to any GO terms. This situation was also identified for the downregulated genes, where among three genes one encoded a hypothetical protein and the others encoded amastin-like surface proteins (LbrM.20.1070 and LbrM.20.4320), but none was related to any GO terms.

Third, we evaluated the DEGs at 24 and 30°C and found that the majority were associated with cellular processes, metabolic processes and biosynthetic processes, not only among the overexpressed genes but also among the downregulated ones. Nevertheless, we also found some upregulated genes related to cell growth and cell proliferation (Table S3) and, interestingly, we found some downregulated genes associated with the aging process, where the promastigotes at 30°C were the most affected (Table S3). In addition, among the upregulated genes shared among these temperatures, there was one encoding a stress-inducible protein, STI1 (LbrM.35.0120), with a similar logFC at both temperatures (data not shown). Moreover, among the downregulated genes, we found ones encoding ABC transporters (LbrM.06.0010, LbrM.11.1000, LbrM.11.1010 and LbrM.15.0930), an amastin surface glycoprotein (LbrM.28.1210), a temperature-dependent protein affecting M2 dsRNA replication (LbrM.21.0910), a heat shock 70-related protein 1 (LbrM.30.2450) and a sodium stibogluconate resistance protein (LbrM.31.1150).

Finally, we evaluated the DEGs shared between 28 and 30°C. Again, we found that the majority of the genes were associated with biological, metabolic and molecular processes, as we reported previously in all of the analyses that we performed. However, we also found some GO terms and genes associated principally with responses to different stimuli. We found some upregulated genes associated with response to nutrient levels and response to starvation, with the logFC value at 30°C being the largest (Table S4), possibly as a consequence of the significant decrease of promastigote density under that condition. In the case of the downregulated genes, we found some related to drug and antibiotic metabolic processes, defense response and regulation of cell proliferation (Table S4). As we reported for the majority of the cases with a decrease in expression, genes encoding an amastin-like surface protein and an amastin surface glycoprotein (LbrM.24.1270 and LbrM.27.0650) were downregulated, while genes encoding ABC transporters (LbrM.15.0820, LbrM.29.0630 and LbrM.29.1750) were also downregulated. Other important genes associated with responses were those encoding a multidrug resistance protein (LbrM.31.1520) and a vitamin B6 photo-protection and homoeostasis-related protein (LbrM.32.2110).

Discussion

The aim of this study was to evaluate the changes at the transcriptomic level in *L. braziliensis* promastigotes under different incubation temperatures to determine the cellular and biological changes in possible future climate change scenarios. In first place we found that the temperature affects the growth curves in *L. braziliensis* where the increase of the temperature is one of the abiotic factors affected as consequence of the global warming. First, we found that the temperature affects the growth curves in *L. braziliensis* where at 30°C had the highest negative effect in parasite concentration. This could be explained by the general response of promastigotes to higher temperatures as a consequence to host change, when it has been found that the *in vitro* promastigotes lost their motility, become rounded and express heat shock proteins (Lawrence & Robertgero, 1985; Toye & Remold, 1989). On the other hand, we illustrated for the first time the response of the growth curves at a lower temperature and we found a continuing growth in parasite concentration such as 26°C but with a lower parasite density. Also, we observed that the BLP at 24°C was later compared to 28 and 30°C. This could be by the effect of low temperatures in microorganism and cells where there are physiology changes like decrease of membrane fluidity, decrease efficiency in transport proteins and a growth with slower rate (Barria, Malecki, & Arraiano, 2013; Nedwell, 1999).

The genes with variation in their copy number and their expression, irrespective of whether this involved over- or under- expression, represented only a small percentage of the DEGs. At 24 and 28°C, only 5.4% of the DEGs could be explained by gene CNVs, and at 30°C the rate was only 3.8%. Thus, in this study, we did not observe a relationship between the gene CNVs and the DEGs as was reported in other studies (Iantorno SA, Durrant C, Khan A et al., 2017). Here, we found that there was a change in the gene expression without a change in the gene CNV in the majority of the DEG. In addition, we found that the temperature of 30°C was the treatment at which more changes occurred compared with the status at the control temperature, since there were more DEGs than in the other two tested treatments. Moreover, as we describe in the results, the chromosome with the highest proportion of DEGs was chromosome 12, which was the only chromosome that did not have variation in the copy number of its genes (Vásquez, unpublished). The last findings support the idea that, in this study, the changes in gene expression could be due to post-transcriptional regulation, potentially mainly involving a trans-splicing reaction of mRNAs and polyadenylation at the 3′-end (Clayton, 2002; Kramer, 2012), mRNA stability, translational control (Clayton & Shapira, 2007; Kramer, 2012; Späth, et al., 2015) and/or mRNA degradation (Clayton & Shapira, 2007), as has been reported in other trypanosomatid studies (Hanke, et al., 2003; Jensen, et al., 2009;

Mcnicoll et al., 2006; Pays, et al., 1989; Queiroz, et al., 2009; Zilka, Garlapati, et al., 2001).

One of the main findings of this study is that, irrespective of the type of analysis performed and described in the results section, in all of the treatments evaluated, we observed a considerable number of genes differentially expressed as a possible direct response to the treatments applied. One of these responses was the overexpression of HSPs, which were upregulated principally at 30°C but less so at 24°C. These upregulated HSPs play important roles in several cellular processes, such as protein folding, assembly, trafficking, activity and degradation. Specifically, the HSPs identified in this study are contained within a complex, since the activator Hsp90 ATPase is fundamental for the activation of HSP90 (Seraphim, et al., 2013), which forms the center of a chaperone complex known as "HSP90 foldosomes". Together with other co-chaperones such as HSP70, HSP40 and stress-inducible protein 1 (Sti1), it is implicated in certain functions such as transduction signaling and cell cycle control (Späth et al., 2015). Thus, it could be inferred that all of the HSPs encoded by DEGs in this study interact with each other and with other gene products such as stress-inducible protein 1 and activator of HSP90 ATPase (also upregulated) to generate a major response. In addition, the HSPs each have other functions, such as under stress functioning in RNA stability and translational control (as previously mentioned, as some of the mechanisms of post-transcriptional regulation) and, in the case of HSP70, in transcript stability (Späth et al., 2015). 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 (Bifeld et al., 2018); and the interaction between HSP90 and Sti1 to promote the fast-growing insect and mammalian host stages of the parasite (Hombach, et al., 2013). Besides the functions of these HSPs, it is known that their synthesis increases at elevated temperatures to protect the organism, as a rapid and efficient cellular response (Hombach, et al., 2014). Thus, we can conclude that, because of the increase of temperature, major synthesis of the HSPs occurs, as we reported here, not only to protect the organism against possible damage, but also as a mechanism of gene expression regulation and adaptation, since they are related to intracellular survival. Therefore, the results obtained here prompt us to conclude that the temperature shifts would not affect negatively parasite biology since the HSPs protect them against possible damage.

Other genes found to be expressed in association with the three treatments were 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 interaction and playing a fundamental role during infection (Cardoso de Paiva et al., 2015) as we mentioned above. Therefore, a reduction in the expression of these genes could affect these processes, as was reported by Cardoso *et al.*, who described that amastin knockdown generated a reduction in the viability of intracellular amastigotes (Cardoso de Paiva et al., 2015). If the decrease in expression of these genes affects the interaction between host and parasite, it would influence a fundamental part of the life cycle, which could in turn generate decreases in the number of parasites and their viability, reducing the number of leishmaniasis cases. In addition, we found two genes arranged in tandem that were upregulated at 24°C and encode to an amastin and a tuzin-like protein. This kind of arrangement was previously reported in *Trypanosoma cruzi*, where it was concluded that the two alternating genes are constitutively transcribed into large polycistronic precursor RNAs and that the tuzin is not processed into a functional mRNA (Teixeira, et al., 1995). However further work is needed to determine the behavior of this gene cluster and its possible implications in *Leishmania,* through the knockdown of these genes.

As we reported several times in the results, other genes that were underexpressed at all the tested temperatures relative to the level at the control temperature were the ones that encode to ABC transporters, which are transmembrane transporters found in a great range of animals. They have been found to play roles in multidrug resistance in mammals and in several microorganisms such as *Plasmodium falciparum*. This characteristic was also reported in *Leishmania*, where at least two classes of ABC transporter are known to be involved in drug resistance (Ouellette et al., 1998).

However, it has also been reported how an ABC transporter is involved in lipid trafficking in the infection process (Leprohon, et al., 2006; Torres et al., 2003). In addition to the ABC transporters, other genes reported here, especially at 30°C, are related to drug processes, such as aquaglyceroporin (AQP1), which increases the sensitivity to SbIII (trivalent antimony, in drugs to treat leishmaniases). A correlation between the accumulation of SbIII and the level of AQP1 RNA was identified (Mandal, et al., 2015; Marquis, et al., 2005), proving that the downregulation of this transporter gene is correlated with drug resistance (Marquis et al., 2005). These results not only indicate a strong relationship between the increase of temperature and drug resistance, but also this and the other responses identified in this study indicate the rapid change in the transcriptome profile in response to an external stimulus. To determine the relationship of drug resistance or host–parasite interaction with increasing temperature, further studies should be undertaken, where the parasites first be subjected to a heat stress and then be subjected to different drugs to evaluate

whether the parasites present any change in their surviving in comparison with parasites without any heat stress treatment. Also, the relationship between the temperature increase and the host-parasite interaction could be evaluate submitting the parasites first to a heat stress and then evaluating their capability to infect macrophages.

In summary, in this study we obtained transcriptome profiles in which the majority of DEGs were associated with biological, cellular and metabolic processes. The interaction between the upregulated and downregulated genes classified under these GO terms determines the changes in promastigote behavior and response to heat stress. For example, as we evidenced at 30°C, there were almost the same proportions of up- and downregulated genes involved in these processes, which could alter the associated functions and as a consequence prompt the appropriate development of the promastigotes. This is evidenced in Figure 1, which shows that, at 30°C, the promastigote cultures were the most affected and showed low cell density.

In conclusion, this study provides evidence that *L. braziliensis* promastigotes exhibit a short-term response to heat stress, generating up- or downregulation of a range of genes associated with different stimuli and other processes such as infection, virulence, survival, growth, drug resistance, cell proliferation and host–parasite interaction. The findings show the great impact of temperature on the transcriptome profiles, as rapid and efficient cellular responses. However, in this study, we cannot draw definitive conclusions on whether the temperature shifts will be advantageous for *L. braziliensis* promastigotes because, while we found some DEGs that favor adaptive processes, other DEGs have opposite effects. Despite that, we found that temperature has a negative effect on the growth curves of *L. braziliensis*, at least for a short term. These findings confirm the important role played by this abiotic factor in the biological processes of the parasite and encourage the planning of more related studies exploring whether temperature has a long-term impact on genome aneuploidy, gene CNVs and whether present other transcriptome profiles. This is important because it is possible that, in a long-term study maintaining the parasites under this stress for several generations, adaptation would be generated through CNVs, as has been reported in drug resistance in *Leishmania*. Finally, it is important highlight that these studies could increase the knowledge of the parasite behavior in a possible and near scenario of the climate change, allowing infer how the leishmaniases epidemiology could change.

Supporting Information

Table S1. Some of upregulated DEG shared between the three treatments. The selection only was based in the GO terms related with some response to stimulus, the genes associated with each term and their change in the expression (Log2FoldChange) are reported at the three temperatures.

Table S2. GO terms of interest for be related with some kind of response to the change in the temperature. The genes associated with each term and their logFC value is reported for the three temperatures assessed.

Table S3. Go terms of interest of the up and down regulated DEG shared between 24 and 30°C, the genes associated to them and their logFC is register.

Table S4. GO terms of interest of the over and under expressed DEG shared between 28 and 30°C, the genes associated to them and their FC is also register.

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