The Earth's rotation forced life to evolve under cyclic day and night environmental changes. To anticipate such daily cycles, prokaryote and eukaryote free-living organisms evolved intrinsic clocks that regulate physiological and behavioural processes. Daily rhythms have been observed in organisms living within hosts, such as parasites. Whether parasites have intrinsic molecular clocks or whether they simply respond to host rhythmic physiological cues remains unknown. Here, we show that *Trypanosoma brucei*, the causative agent of human sleeping sickness, has an intrinsic circadian clock that regulates its metabolism in two different stages of the life cycle. We found that, in vitro, ~10% of genes in *T. brucei* are expressed with a circadian rhythm. The maximum expression of these genes occurs at two different phases of the day and may depend on a post-transcriptional mechanism. Circadian genes are enriched in cellular metabolic pathways and coincide with two peaks of intracellular adenosine triphosphate concentration. Moreover, daily changes in the parasite population lead to differences in suramin sensitivity, a drug commonly used to treat this infection. These results demonstrate that parasites have an intrinsic circadian clock that is independent of the host, and which regulates parasite biology throughout the day.
Figure 1 | *T. brucei* has a circadian transcriptome in two stages of the life cycle, mammalian bloodstream and insect procyclic forms. a, Populations of parasites were entrained to 12 h:12 h temperature intervals for three days, after which they were kept under alternating conditions or released into constant conditions for 2 days. During these 2 days, RNA was collected every 4 h for RNAseq (see Methods and Supplementary Fig. 3). b, Gene expression heatmap views of temperature-entrained cycling genes of bloodstream and procyclic forms. Each row represents a gene, ordered vertically by phase, determined by ARSER. N = total number of cycling genes identified. See Supplementary Data 1–2. c, Phase distribution of cycling genes entrained by temperature. The phase of each gene’s rhythm across the day is represented in a histogram plot (top) and a rose plot (bottom). The mean circular phase of the different phase clusters is indicated by an orange dashed line. d, Venn diagram of number of cycling genes identified in temperature-entrained cycling and constant conditions for bloodstream (left) and insect procyclic (right) forms. e, Period distribution of genes cycling in alternating (teal) and constant (grey) temperature in both life-cycle stages: bloodstream forms (left) and insect procyclic forms (right). The total area under the curve is one for each condition. Under alternating temperature conditions, the period of cycling genes is centred around 24 h, while under constant temperature, the distribution of the period of cycling genes is broader. The dashed line indicates the expected entraining period of 24 h for alternating conditions.
Figure 2 | Circadian expression is temperature-compensated and detected in vivo during a mouse infection. a, Genome browser views of RNAseq coverage from bloodstream-form parasites in temperature-entrained conditions for two genes: Tb927.10.16100, FK506-binding protein (FKBP)-type peptidyl-prolyl isomerase, putative, and Tb927.1.4830, phospholipase A1 (genes represented in teal), out of the ∼1,100 genes cycling. The coding sequence (CDS) is represented as a green rectangle, and intergenic regions as grey dotted lines. Read coverage is shown in black as reads per million total reads (RPM) across 48 h. b, RPKM (reads per kilobase of transcript per million mapped reads) quantification of RNAseq read coverage and circadian algorithm fits. ARSER fit is represented by a dark grey dashed line, JTK_CYCLE in teal and Fisher’s G-test in orange. Represented genes are the same as above: FKBP (JTK_CYCLE, ARSER and Fisher’s G-test P < 0.01) and phospholipase A1 (ARSER and Fisher’s G-test P < 0.05). c, Period of oscillation of 127 common cycling genes at constant temperatures of 28 °C (procyclic forms) and 37 °C (bloodstream forms) (left) and distribution of the estimated temperature coefficient (Q_{10}) for the period of the 127 common cycling genes (right). d, Expression of two representative cycling genes in vitro (left) and in vivo (right) (9 genes cycled in vivo out of the 11 genes tested; for more examples see Supplementary Fig. 5). Transcript values in vitro were retrieved from RNAseq analysis of the bloodstream-form transcriptome under constant temperature. To measure transcript levels in vivo, RNA was extracted from parasites in the blood of infected mice. Transcript levels of proline dehydrogenase (Tb927.7.210) and putative amino acid (a.a.) transporter (Tb927.8.7650) were normalized to non-cycling transcripts of zinc finger protein 3 (ZFP3, Tb927.3.720, teal) and acidic phosphatase (Tb927.5.610, dark teal). N = 18 (three mice per time point). Error bars represent standard error. Genes were found to be cycling significantly by ARSER, P < 0.05.
in both bloodstream-form data sets. This shows that the time of sample collection is a key factor in these samples (Supplementary Fig. 3c). Circadian analysis of temperature-entrained bloodstream forms (Fig. 1 and Supplementary Data 1) revealed that ∼1,100–1,500 transcripts cycled, with 1,490 genes (∼15% of genome) oscillating under alternating conditions and 1,092 genes (∼11% of genome) under constant temperature (Fig. 1b and Supplementary Figs 3 and 4). Two examples of cycling transcripts are shown in Fig. 2a,b.

Unlike what we observed when bloodstream-form parasites were subjected to light/dark cycles, upon temperature entrainment the permutation test revealed that the number of cycling genes identified in the correct sampling order was significantly higher than when sampling order was randomly permuted (FDR < 0.05, Supplementary Fig. 2), indicating that we are detecting transcript oscillations above background noise and therefore the temperature-entrainment protocol can synchronize the bloodstream-form parasite population. Are these oscillations dependent on temperature entrainment, or would they be detected in cultures without entrainment? To confirm that temperature entrainment was required, we compared the cycling genes identified under two constant conditions (both at 37 °C in darkness): either after temperature entrainment or light/dark cycling, suggesting that the circadian clocks of the parasites in the population remained asynchronous. Therefore, the constant-darkness data set is a fair proxy of ‘no entrainment’ conditions. If a transcript oscillation is dependent on entrainment, this gene should only oscillate after temperature entrainment. Indeed, 1,050 of 1,092 genes (96%) exclusively oscillate after temperature entrainment, suggesting that those transcript oscillations are dependent on temperature entrainment.

The majority of the oscillating genes have a maximum expression at environmental Zeitgeber Time (ZT) ZT8 and ZT20 (Fig. 1c), a bimodal distribution that is also typical in other eukaryotes. Under constant temperature, phases are shifted ∼2–3 h, corresponding to Circadian Time (CT) CT11 and CT22 (Fig. 1c). As observed in
other systems\(^9,10\), we found that the oscillatory transcriptome is divergent between alternating and constant conditions and three categories of genes were identified: (1) genes oscillating in alternating conditions only (temperature-driven and clock-independent) (1,243 genes of bloodstream forms); (2) genes oscillating in both alternating and constant conditions (clock-driven genes) (247 genes); and (3) genes oscillating in constant conditions only (genes whose cycling is suppressed or masked during entrainment conditions) (845 genes) (Fig. 1d). A hallmark of circadian rhythm predicts that if parasites are entrained to temperature cycles, we should expect that in the presence of entrainment the period of the cycling transcripts will be precisely 24 h and when the stimulus is removed (constant conditions) the period should remain close to 24 h, but not as precisely. This is indeed what we observed. The median period of the 247 genes cycling under alternating temperature was centred around 24 h, and was shorter at constant temperature. The variance of the period lengths was significantly tighter under alternating temperature than under constant conditions (Kolmogorov–Smirnov test, \(P < 2.2 \times 10^{-16}\), Fig. 1e, and Supplementary Fig. 6). These results indicate, once again, that entrainment of these transcript oscillations is dependent on temperature cycles.

Among the mammalian bloodstream forms, there are two distinct stages of the life cycle that differ transcriptionally: the replicative bloodstream slender form and the short-lived cell-cycle arrested bloodstream stumpy form. Slender forms differentiate into the transmissible stumpy form via a mechanism of quorum sensing typically triggered in vitro at densities higher than \(\sim 1 \times 10^6\) parasites per ml (ref. 11). These two life-cycle stages are transcriptionally
and metabolically different, which could add noise to our circadian studies. To obtain cultures with replicative slender forms only, we kept each culture below 1 × 10^6 parasites per ml. To further confirm that our parasite population was primarily composed by slender forms, we repeated the temperature entrainment with a GFP::PAD1′utr reporter cell line, in which a green fluorescent protein (GFP) gene is followed by a PAD1′ untranslated region (UTR) that confers maximum expression in stumpy forms. Using fluorescence-activated cell sorting (FACS), we assessed GFP expression and the cell cycle profile by propidium iodide staining. We found that ∼95% of our cultures were GFP-negative, with no apparent G1-cell-cycle arrest, indicating that most parasites were in the slender form at all time points. We conclude that slender forms alone can be responsible for the observed circadian gene expression.

When the host is bitten by a tsetse fly, T. brucei stumpy forms differentiate into procyclic forms that are adapted to live in the midgut of the fly where the temperature is much lower (∼28 °C). Even though the transcriptome of bloodstream and insect procyclic forms is ∼30% distinct, we tested whether insect procyclic trypanosomes share a circadian transcriptome temperature when entraining with 23 °C/28 °C cycles (Fig. 1a and Supplementary Fig. 3). As seen with the bloodstream forms, an unbiased comparison of the 13 samples in each data set using hierarchical clustering and PCA showed that a cyclic pattern component accounted for 20–27% of the total variance, being the first component in both data sets. In these trypanosomes, we identified 1,123 genes cycling under alternating conditions and 854 genes cycling endogenously, that is, after temperature entrainment was removed (Fig. 1a–d, Supplementary Data 2 and Supplementary Figs 3 and 4). Of these, 127 genes (∼1–2% of transcriptome) oscillate in both life-cycle stages, while 965 are specific to bloodstream forms and 727 to procyclic forms (Supplementary Fig. 5a). The analysis to confirm if oscillations are dependent on temperature entrainment cannot be done for the insect procyclic forms, because we did not perform light/dark entrainment in this life-cycle stage.

Overall, these data show that in each of the two stages of the life cycle of T. brucei, ∼10% of the transcriptome undergoes circadian oscillations, suggesting that having a circadian rhythm might have conferred an evolutionary advantage throughout the parasite life
cycle. The fact that most cycling genes differ between the two stages indicates that the circadian clock can sense and adapt to the different host environments, another hallmark of circadian clocks.

**Daily transcriptome is temperature compensated**

We have shown, above, that transcript oscillations are entrained by temperature cycles. Another canonical property of circadian clocks is temperature compensation: the ability of the period of a rhythm to remain relatively constant at various physiologically permissive temperatures. The $Q_{10}$ temperature coefficient measures the rate of change in a biological system when the temperature is increased by 10 °C. Whereas the kinetics of most biological systems double or triple when increasing by 10 °C (and thus $Q_{10}$ within 2 and 3), the rate of biological reactions regulated by a circadian rhythm does not change with a temperature increase ($Q_{10}$ of $\sim 1$). To test whether T. brucei transcript oscillations are temperature-compensated, we compared the periods of 127 genes that oscillate at constant 28 °C and constant 37 °C (Supplementary Fig. 5a). We observed no significant changes, and the average $Q_{10}$ was 0.99 ± 0.13 (standard deviation, s.d., Fig. 2c) showing that the circadian clock in T. brucei is temperature-compensated.

**Parasite gene transcripts also cycle in a mouse infection**

As the identification of the trypanosome circadian transcriptome was performed in parasites grown in culture, we next tested whether transcript oscillations could also be found in vivo, that is, in parasites from an infected mouse. For this, we collected blood every 4 h, and RNA was extracted and subjected to real-time quantitative PCR (qPCR). We confirmed that transcript levels of most genes also cycled in vivo (Fig. 2d and Supplementary Fig. 5d; 9 transcripts cycled out of 11 tested), including proline dehydrogenase and a putative amino acid transporter.

When we compare the circadian transcriptome of T. brucei (bloodstream and procyclic) with other organisms, we note that T. brucei has fewer cycling transcripts than a mouse liver (~10% versus 20%, respectively)16, but it has significantly more than those identified in human and mouse immortalized cell lines (0.1–1.2%)17,18. The amplitude of the oscillations in bloodstream forms is on average ~1.4-fold (~40% difference), which is within the range of what is described (~2.3-fold in the mouse liver16 and low in cell lines17,18). This lower amplitude found in culture systems may be due to asynchrony within the cell population (diluting the maximum and minimum expression levels), as well as the absence of additional entraining cues present in vivo. In fact, for the genes we studied in vivo, we observed on average higher amplitude than in vitro (1.86-fold ± 0.06 and 1.49-fold ± 0.1 s.d., respectively, P < 0.001, two-tailed Mann–Whitney test). The fact that the Gim5A transcript (Supplementary Fig. 5d) showed opposite phases when measured by in vitro RNAseq and in vivo real-time PCR suggests, once more, that there may be additional entraining signals in vivo that differentially affect the amplitude and phase of gene expression19.

Taken together, these results show that host physiological rhythms (in vivo) and temperature (in culture) are capable of synchronizing T. brucei parasites and that the transcriptome circadian oscillation is driven by an endogenous clock.

**Circadian regulation is post-transcriptional**

Although, in eukaryotes, the circadian timekeeping mechanism is based on a transcription/translation feedback loop model, recent studies have shown that post-transcriptional and post-translational steps impose further levels of circadian regulation. T. brucei and other Kinetoplastida are peculiar eukaryotes, as most of the genome is organized into polycistronic units (PCUs) that are constitutively transcribed; as a result, gene expression is mainly regulated post-transcriptionally21,22. To determine whether T. brucei circadian gene expression is also post-transcriptionally regulated, we tested whether cycling genes clustered in specific PCUs. We found that cycling genes show a uniform distribution among most PCUs (with a similar proportion of cycling and non-cycling genes, Kolmogorov–Smirnov $P > 0.1$), with no bias for a specific position within a PCU (Kolmogorov–Smirnov test, $P > 0.5$), nor enrichment for genes peaking at a specific phase (Kolmogorov Smirnov test, $P > 0.1$, Fig. 3a–d and Supplementary Fig. 8). The fact that co-transcribed genes can either not cycle or cycle with maximum expression at opposing phases indicates that the timekeeping mechanism used by T. brucei is primarily based on post-transcriptional regulation, which is a different mechanism of timekeeping from other eukaryotes.

**Circadian transcriptome is not dependent on the cell cycle**

In culture, T. brucei parasites replicate every ~6–7 h in bloodstream forms and every ~11–12 h in procyclic forms. Although circadian oscillations have a period of ~24 h, we wondered whether temperature entrainment could synchronize the cell cycle of parasites and, as a result, cell cycle could be contributing to the circadian oscillations of transcripts. To rule out this possibility, we temperature-entrained T. brucei bloodstream cultures and collected cells throughout the day to measure DNA content. Not surprisingly, we observed that parasite cultures under alternating conditions grew more slowly than under constant 37 °C (7 h 33 min versus 6 h 53 min doubling time, respectively). However, the frequency of dividing cells in the population was constant throughout the day (~30% in G2/M), suggesting that parasite cell division was not synchronized to occur at a certain time of the day (Fig. 3e). Furthermore, among all cycling transcripts, we detected no enrichment of cell-cycle-associated genes (Supplementary Table 1), as illustrated by the expression profile of DNA topoisomerase II and cdcl2-related kinase 3 (Fig. 3f).

Together, these data indicate that the cyclic pattern of the T. brucei bloodstream transcriptome is not dependent on the cell cycle.

**Metabolism changes throughout the day**

To explore the biological relevance of a circadian clock in T. brucei, we performed a temporal gene ontology (GO) analysis. We assigned the genes that cycled under constant temperature conditions in bloodstream and procyclic forms into 12 groups based on the phase of maximal expression, and evaluated the enrichment of GO terms (Fig. 4a and Supplementary Data 4). We found that 95% of cycling GO terms are enriched in only one phase cluster in the bloodstream form (93% in insect procyclic forms), suggesting that specific cellular processes are upregulated at different times of the day. One such process is carbohydrate metabolism, in which 13 of 31 genes annotated to this GO term peaked expression at opposing phases of the day. One such process is carbohydrate metabolism, in which 13 of 31 genes annotated to this GO term peaked expression at opposing phases of the day. One such process is carbohydrate metabolism, in which 13 of 31 genes annotated to this GO term peaked expression at opposing phases of the day. One such process is carbohydrate metabolism, in which 13 of 31 genes annotated to this GO term peaked expression at opposing phases of the day. One such process is carbohydrate metabolism, in which 13 of 31 genes annotated to this GO term peaked expression at opposing phases of the day. One such process is carbohydrate metabolism, in which 13 of 31 genes annotated to this GO term peaked expression at opposing phases of the day. One such process is carbohydrate metabolism, in which 13 of 31 genes annotated to this GO term peaked expression at opposing phases of the day. One such process is carbohydrate metabolism, in which 13 of 31 genes annotated to this GO term peaked expression at opposing phases of the day.
time-dependent sensitivity to H$_2$O$_2$ treatment, which was 2.6-fold (Fig. 5b). Thus, we conclude that, during the day, bloodstream-form feeding regime. Because the microbiome appear to be driven by the host and by its extracellular and it can be controlled easily, it is an ideal system to study whether a pathogen has an intrinsic mechanism to keep time. By analysing the transcriptome of parasites grown in vitro after entrainment to temperature, we found that T. brucei has a circadian oscillating transcriptome.

What is the difference between a circadian rhythm and a response to a subtle heat shock? A circadian clock has three bona fide hallmarks that we found in trypanosomes: it has a free running period of ~24 h under constant conditions, it is entrainable, and it is temperature-compensated. The first hallmark was met when we found ~1,100 genes that oscillate under constant conditions with a free running period of ~24 h. Entrainment is supported by two observations. First, 96% of the temperature-entrained transcript oscillations in bloodstream forms are absent under light/dark cycle conditions, where entrainment did not appear to occur (Supplementary Fig. 5b). Second, the period and phase of T. brucei oscillating genes were close but somewhat different between alternating and constant conditions (Fig. 1c,e). The hallmark of temperature compensation was demonstrated by the fact that the period of oscillating genes remains ~24 h when parasites are at 37 or 28 °C (Fig. 2c).

In this study we have shown that replicative slender bloodstream-form and procyclic-form parasites have circadian transcriptomes that regulate multiple metabolic pathways. In addition, it is possible that, with a circadian clock, parasites are better prepared to escape the host immune response, which is itself under circadian control. A circadian clock may also be important for transmission success; because the tsetse fly has a daily biting pattern, having transmissible forms ready to match when the vector tsetse is more likely to bite would be an advantage. Also within tissues, parasites with a timekeeping mechanism could anticipate fluctuations in nutrient availability within the interstitial spaces. Although it is likely that the transmissible non-replicative stumpy form also have circadian rhythms, this still requires further analysis.

Transcriptomic oscillations result in cyclic changes in the parasite population with a ~24 h period, primarily at the metabolic and for parasites diverse in the vector, parasites divided in the morning are different from those in the evening, an unprecedented concept in disease-causing pathogens. These rhythms are probably an important adaptation, because, in vivo, their host environment (mammal or insect) also undergoes circadian changes.

Many questions remain to be answered in the future. What is the daily transcriptome of bloodstream forms in vivo? Due to the complexity of the host environment, we would expect to identify a larger number of parasite oscillating genes and with more robust amplitudes. What are the core clock genes that drive these oscillations in gene expression? Discovering this will be essential not only to understand the molecular mechanism underlying circadian rhythm in trypanosomes, but also to use it as a tool to demonstrate which of the oscillations detected in vivo are a result of an intrinsic parasite clock and which are imposed by the host. Finally, it will be interesting to test whether light and dark cycles can entrain procyclic forms, as these parasites are more exposed to light while in the tsetse fly.

This study demonstrates the potential of high-throughput approaches for identifying circadian patterns in the transcriptome of non-model organisms and it provides a foundation for the search of the master regulators of this process in T. brucei and for the search for endogenous clocks in other important infectious agents such as the malaria parasite.

### Methods

**Ethics statement.** All animal care and experimental procedures were performed in accordance with University of Texas Southwestern Medical Center (UTSW) IACUC guidelines, approved by the Ethical Review Committee at the University of Southwestern Medical Center and performed under the IACUC-2012-0012 protocol.

**Parasites and culture conditions.** T. brucei AnTat 1.1, a ploimorphic clone derived from an EATRO1125 clone, was originally isolated from the blood of *Glossina morsitans* in Uganda. For all the experiments we used AnTat 1.1, a transgenic line encoding the tetracyclin repressor and 17 RNA polymerase.

Bloodstream forms were grown routinely in HM1-41 at 37 °C in 5% CO$_2$ (ref. 35). For all RNAseq experiments, individual cultures of parasites were prepared, adjusting the initial parasite density so that parasite cultures would be at 1 x 10$^7$ parasites per ml at each collection time point. Parasite numbers were calculated using a haemocytometer. Synchronizations were carried out for three days in alternating temperature or light conditions. For the bloodstream-form temperature RNAseq experiment, culture flasks were moved every 12 h between incubators either at 32 or 37 °C or were kept at constant 37 °C. In the bloodstream-form light/dark entrainment RNAseq experiment, a warm white light-emitting diode 3 W lamp was used to illuminate the cultures inside the incubator. The temperature was kept at 37 °C and fluctuations were monitored and shown to be less than 0.1 °C. At the end of day 3 (72 h), culture flasks were split into alternating or constant conditions groups. RNA samples were collected every 4 h for 2 days (a total of 13 samples per condition, with the second cycle acting as a biological replicate).

Differenitation of bloodstream forms to procyclic forms was induced by adding 6 mM cit-aconitate to differentiating trypanosome medium and by reducing the temperature to 28 °C. The newly differentiated procyclic cultures were maintained as described previously. Differentiation was assessed by EP procyclin expression, using an anti-T. brucei procyclin, fluorescein isothiocyanate mouse IgG1 (Cedarlane Labs). For the procyclic-form RNAseq experiment, culture flasks were moved every 12 h between incubators either at 23 or 28 °C, or kept at constant 28 °C. For a schematic representation see Fig. 1a and Supplementary Figs 1 and 2. To confirm that the bloodstream-form cultures were primarily composed of slender forms, we used a GFP-PAD1utr reporter cell line, in which a GFP gene is followed by a PAD1 UTR that confers maximum expression in stumpy forms.

**Transcriptome sequencing (RNAseq).** RNA was isolated from ~1 x 10$^7$ T. brucei cells (density of ~1 x 10$^6$ per ml) with TRIzol reagent according to the
manufacturer’s instructions (Life Technologies). Total RNA (1 μg) was enriched for mRNA with PolyA-tract by using the TrueSeq RNA Sample preparation protocol (Illumina). RNA-sequence of libraries was performed on the HiSeq2000 platform (Illumina) with 50 bp reads, according to the manufacturer’s instructions, by the UTSW McDermott Next Generation Sequencing Core and Beijing Genomics Institute (BGI). Read quality was assessed using the FASTQC quality control tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The SolexaQA suite of programs was used to trim raw reads to their longest contiguous segment above a threshold of v ≤ 2 – 1. The number of reads mapping to each gene was determined and then normalized to RPKM (reads per kilobase of transcript per million mapped reads) (estimating a high-expressed VSG gene from the calculation) using the R software environment and the packages GenomicAlignments39, Biostrings40 and rtracklayer41 from Bioconductor.

The number of uniquely mapped reads in each sample (~20 million reads) was enough to detect the expression of more than 80% of annotated T. brucei genes (five or more reads mapped over coding sequence) and it was verified that this number does not increase significantly with added depth. The distribution of RPKM values in each sample was plotted, and was verified to be similar across all samples.

Time-series analysis for circadian cycling. Hierarchical clustering analysis was performed using Spearman correlation to centre transformed RPKM values (Supplementary Figs 1b and 3c,d) were produced in the R software environment using the function heatmap.2 from the gplots package.42 PCA was performed on centred and log-transformed RPKM values using the function princomp. RNA cycling was assessed using three programs: GeneCycle, which implements Fisher’s G-test, ITK_CYCLE (ref. 45) and ARSER (ref. 46). For Fisher’s G-test and ITK_CYCLE analyses, RPKM data were detrended by linear regression. A gene was considered cycling if two of three programs detected periodic expression with a threshold of P ≤ 0.05 and mean expression higher than 10 RPKM. This cutoff was defined by assessing the coefficient of variation in relation to the mean expression across all time points. The amplitude, period and phase were determined as described above. The heatmaps of phases in Fig. 1b and Supplementary Fig. 1d plotted the Z-score transformed RPKM values, ordered by the phase determined by ARSER. The peaks of expression phase distributions were determined by fitting a mixed von Mises–Fisher model to the bimodal phase distributions using the R software environment and the mvnMF package,42 extracted the modes of the two von Mises–Fisher distributions.

To determine the FDR of identification of cycling genes, the time points of collection were randomized and the number of cycling genes assessed. These permutation tests were run 10,000 times for each of the six data sets.

Chromosome distribution of cycling genes. The uniform distribution of cycling genes among PCUs was tested by comparing the distribution of proportions of cycling/non-cycling genes per PCU with a distribution obtained by randomly sampling the same number of genes from the genome 10,000 times. To test if cycling genes displayed a bias in their positioning within PCUs, the distance of each gene to its nearest centromere was calculated, and the distribution of these distances for cycling genes only and for all genes were then compared. To test if cycling genes within a PCU tend to peak at the same phase, we compared the distribution of proportions of each phase cluster within PCUs with a random distribution obtained by randomly permuting the cluster assignment of cycling genes 10,000 times. For these three analyses, significance was assessed by a Kolmogorov–Smirnov test.

Mice infection and real-time quantitative PCR analysis. The infections of 18 six- to ten-week-old wild-type male C57BL/6j mice (UT Southwestern Medical Center Mouse Breeding Core facility) were performed by intraperitoneal (i.p.) injection of 2,000 T. brucei AnTat 1.1 parasites.43 Before infection, T. brucei cryostatblades were thawed, and parasite viability and numbers were assessed by mobility under a microscope. Mice were individually housed in activity wheel-equipped cages under light/dark (12:12 h) for 7 days, after which animals were kept in dark conditions. Chow and water were available ad libitum. Lokolocality activity was recorded and analysed using ClockLab software (Actimetrics) to determine the circadian phase, as previously described,44 for each animal, on day 20 post-infection. Mice were assigned to six time points of collection, and every 4 h, three mice were killed and terminal cardiac blood samples were collected. All mice included in the study were infected as monitored by measuring parasitaemia. Gross locomotor activity was determined based on early in vivo experiments using this experimental model, and animals were randomly selected for each time point of collection. All quantitative analyses were performed in a blinded manner. RNA was extracted with TRIZol LS according to the manufacturer’s instructions (Life Technologies), and reverse transcription and real-time PCR were performed as described previously.44 Primer efficiencies were determined using standard curves with 3-log10 coverage. Transcript levels were normalized to genes zinc finger protein 3 (ZFP3, T9273.5:720) and a cellular apoptosis regulator (T9275.6:610), whose expression remained constant in both temperature and light alternating conditions. Primer sequences are listed in Supplementary Table 2.

Assessment of metabolic activity and cell-cycle stage. Bloodstream-form parasites were cultured and entrained by temperature as described above for the RNAseq experiment. Samples were collected every 4 h throughout the day. For metabolic activity assessment through measurement of ATP concentration in the parasite population, parasitaemia was assessed, and metabolic activity was measured according to the manufacturer’s instructions by CellTiter-Glo Luminescent Cell Viability Assay (Promega) from 1 × 10⁶ parasites centrifuged and resuspended in 25 μl trypansosome dilution buffer (TDB, 5 mM KCl 80 mM NaCl, 1 mM MgSO₄, 20 mM Na₂HPO₄, 2 mM NaH₂PO₄, 20 mM glucose, pH 7.7). ATP concentration was measured in two independent experiments from a minimum of 30,000 events. The intensity of red fluorescence was measured using a FACScalibur flow cytometer (BD Biosciences), and data were analysed using FlowJo.

Functional analysis of cycling genes. Cycling genes identified by RNAseq were clustered into 12 groups (CT0–CT2, CT2–CT4, and so on) based on their expression patterns. GO terms from centromere to telomere were determined by moving the genome carrying cycling genes 10,000 times. For these three analyses, significance was assessed by a Fisher exact test, then plotted as a heatmap in Fig. 4a. Manually curated GO terms were defined when both statistical tests show enrichment (P < 0.05) and more than three genes annotated to a GO term were cycling with the determined phase (Supplementary Data 4).

Suramin and H₂O₂ sensitivity assay. Bloodstream-form parasites were cultured and entrained by temperature as described above for the RNAseq experiment. Every 4 h, parasites were collected from exponential-phase cultures, counted, and plated in 96-well flat-bottom microtitre plates at a parasite density of 10,000–20,000 cells per well. Serial dilution concentrations (1:3) of suramin (Sigma) were added. The compound was applied in triplicate at eight concentrations and incubated for 24 h at 37 °C. For the oxidative stress experiment, H₂O₂ (Sigma) at six different dilutions was tested (1:10) and parasites were incubated for 1 h at 37 °C. Alamar Blue (Sigma) was used to determine cell viability by adding at 10% of the well volume followed by 4 h incubation at 37 °C. Fluorescence was measured with excitation/ emission of 530/590 nm, and the percentage of live cells calculated by normalizing to non-treated parasites. Calculation of IC₅₀ values was carried out by a four-parameter nonlinear curve fit (GraphPad Prism), and significance was assessed by the extra sum-of-squares F-test. H₂O₂ sensitivity was tested in two independent experiments from a total of six biological replicates, and data was tested in three independent experiments, and IC₅₀ values are shown as the mean of those experiments (N = 9).

Data availability. The RNAseq data sets are often referred to as CW for cold/warm alternating conditions, WW for warm/warm constant conditions, LD for light/dark alternating conditions and DD for dark/dark constant conditions. RPKM and circadian oscillation analyses of all these data sets are available in the present manuscript in Supplementary Data 1–3. RNAseq data sets generated as part of this study have also been submitted to the ArrayExpress database ([https://www.ebi.ac.uk/arrayexpress]) under accession no. E-MTAB-4952.

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