

# Catalase compromises the development of the insect and mammalian stages of *Trypanosoma brucei*

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## Keywords

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Catalase is a widespread heme-containing enzyme, which converts hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to water and molecular oxygen, thereby protecting cells from the toxic effects of H<sub>2</sub>O<sub>2</sub>. *Trypanosoma brucei* is an aerobic protist, which conspicuously lacks this potent enzyme, present in virtually all organisms exposed to oxidative stress. To uncover the reasons for its absence in *T. brucei*, we overexpressed different catalases in procyclic and bloodstream stages of the parasite. The heterologous enzymes originated from the related insect-confined trypanosomatid *Crithidia fasciculata* and the human. While the trypanosomatid enzyme (cCAT) operates at low temperatures, its human homolog (hCAT) is adapted to the warm-blooded environment. Despite the presence of peroxisomal targeting signal in hCAT, both human and *C. fasciculata* catalases localized to the cytosol of *T. brucei*. Even though cCAT was efficiently expressed in both life cycle stages, the enzyme was active in the procyclic stage, increasing cell's resistance to the H<sub>2</sub>O<sub>2</sub> stress, yet its activity was suppressed in the cultured bloodstream stage. Surprisingly, following the expression of hCAT, the ability to establish the *T. brucei* infection in the tsetse fly midgut was compromised. In the mouse model, hCAT attenuated parasitemia and, consequently, increased the host's survival. Hence, we suggest that the activity of catalase in *T. brucei* is beneficial *in vitro*, yet it becomes detrimental for parasite's proliferation in both invertebrate and vertebrate hosts, leading to an inability to carry this, otherwise omnipresent, enzyme.

## Introduction

The protistan parasite *Trypanosoma brucei* is the causative agent of African sleeping sickness, a fatal disease of humans and livestock, which is endemic to sub-Saharan Africa.

*Trypanosoma brucei* is transmitted to its mammalian host by an infected tsetse fly *Glossina* spp. via blood feeding [1]. To prosper in both the insect vector and

the mammalian host, *T. brucei* has evolved a complex dioxenous life cycle that comprises several developmental stages [2]. The most commonly studied ones are the procyclic stage (PCF) in the insect's midgut and the bloodstream stage (BSF) in the mammalian host [3]. When transferred from one host to another and during the interstage transitions between different host tissues

## Abbreviations

BSF, bloodstream stage; cCAT, *Crithidia fasciculata* catalase; hCAT, human catalase; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; PCF, procyclic stage; ROS, reactive oxygen species; Tet, tetracycline.

and compartments, the parasite is exposed to a plethora of oxidative stresses [4,5].

Reactive oxygen species (ROS) are free radicals, such as superoxide anion ( $O_2^{\bullet-}$ ), hydroxyl radical ( $\bullet OH$ ), per hydroxyl radical ( $HO_2\bullet$ ), or hydrogen peroxide ( $H_2O_2$ ), that are produced from the redox reactions [6]. It is known that ROS play a substantial role in mediating the host defense against a broad range of pathogens [7]. For example, it has been shown that ROS facilitates apoptosis in PCF *T. brucei* [8]. In tsetse flies, the increased level of  $H_2O_2$  was detected in the proventriculus, following a trypanosome infection [9]. Also, proteins involved in oxidative stress protection were upregulated in the midgut of the infected flies [10]. Analogously, trypanosome infections in mammals are associated with increased levels of ROS in the serum [11]. Trypanosomes have a whole range of ROS detoxification systems, such as trypanothione, glutathione, trypanredoxins, peroxiredoxins, or superoxide dismutases, except for catalase, which is missing from all their genomes analyzed to date [12–14]. In many species, including humans, the catalase contains a distinctive peroxisomal targeting signal sequence [15]. Trypanosomes have evolved specialized peroxisomes, called glycosomes which sequester most components of the glycolytic pathway [16]. The catalase is present in almost all extant organisms exposed to oxygen [17]. Still, some free-living eukaryotes, such as secondary algae (Cryptophyta, Haptophyta, and Chlorarachniophyta), hematozoan Apicomplexa, and the bodonid and euglenid flagellates, lack catalase in their genomes [18,19]. Recently, it was shown that the distribution of catalase in the kinetoplastid protists of the family Trypanosomatidae is particularly idiosyncratic [19]. These obligatory parasites evolved from their free-living relatives and either use a single insect host (monoxenous life cycle) or circulate between insect and vertebrate or plant hosts (dixenous life cycle) [20,21]. Catalase was shown to be restricted to the monoxenous trypanosomatids of the genera *Crithidia*, *Leptomonas*, and *Lotmaria* [22–24] and appears to be regulated differently in various species. In *Leptomonas seymouri*, the expression of catalase is upregulated at elevated temperatures, while in *Crithidia thermophila*, it remains stable [25,26]. On the other hand, catalase is absent from the members of the dixenous genera *Leishmania* and *Trypanosoma* pathogenic for humans and other mammals [27]. While the reasons behind this remain unknown, it was speculated that the enzyme might prevent sensing minute changes of the  $H_2O_2$  levels, which are known to be important for differentiation [19,28].

A crucial role of  $H_2O_2$  in differentiation and virulence has been demonstrated for another

trypanosomatid, *Trypanosoma cruzi*, the intracellular parasite causing Chagas disease [29,30]. Importantly, when catalase from *Escherichia coli* was heterologously expressed in *T. cruzi*, the ability of the parasite to signal oxidative stress was altered [31]. Levels of the *T. cruzi* antioxidant enzymes, such as trypanothione reductase and superoxide dismutase, were decreased and increased, respectively. The presence of the bacterial catalase also affected the fitness of *T. cruzi in vivo*, with elevated proliferation rate of the promastigotes in the insect vector *Rhodnius prolixus*, but no significant influence on parasite virulence in the vertebrate host. Overall, this study documented a marginally positive impact of a heterologous catalase expression on parasite's fitness [31].

This study aimed to investigate the impact of catalase expression in an extracellular parasite *T. brucei*. In the BSF and PCF trypanosomes, we have tested catalases derived from either a closely related trypanosomatid *Crithidia fasciculata* or a human. Although both enzymes appear to be beneficial *in vitro*, their overall impact *in vivo* is negative. Combined, our data confirm that the catalase activity is incompatible with the dixenous life cycle of *T. brucei* and, likely, other members of the genus *Trypanosoma*.

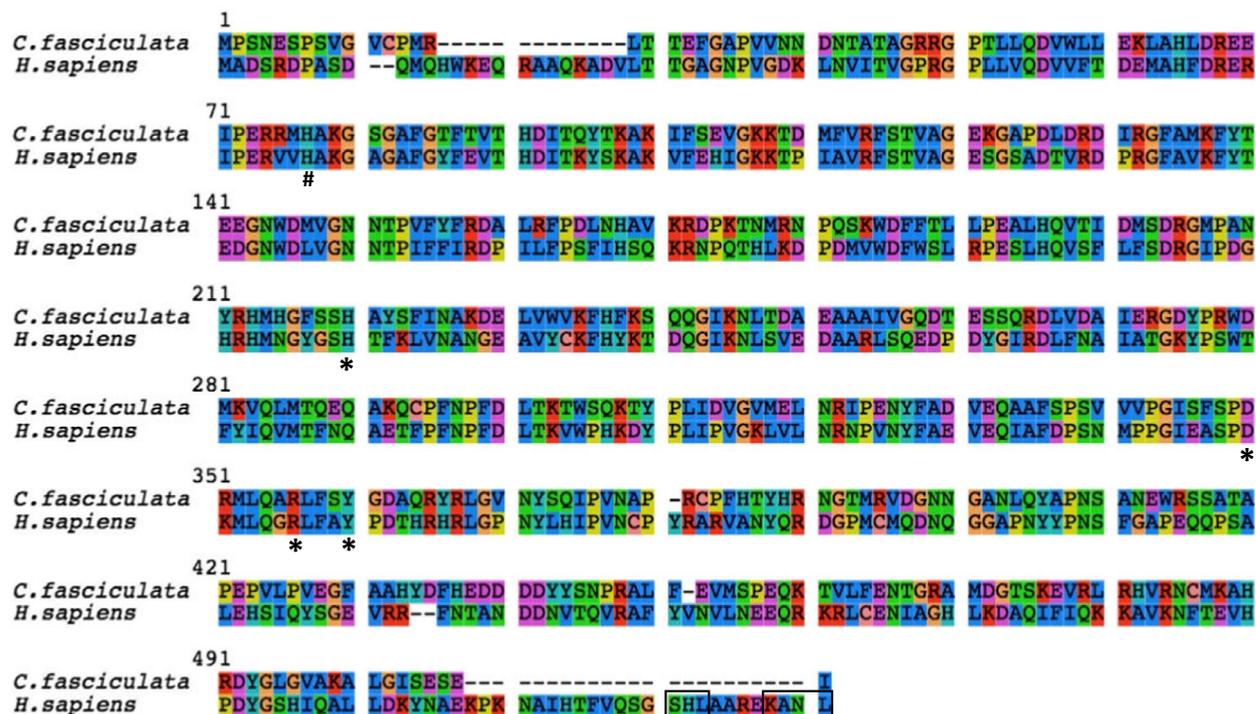
## Results

### *Crithidia fasciculata* and human catalases show a high level of conservation

In this study, two catalase genes, cCAT from *C. fasciculata* and hCAT from human, were used for heterologous expression in *T. brucei*. The cCAT protein is 52% identical to its hCAT orthologue. The active site of catalase is formed by a heme moiety bound to His<sub>75</sub> (hereafter, amino acids are numbered as in the hCAT). Several residues (His<sub>218</sub>, Asp<sub>348</sub>, Arg<sub>354</sub>, and Tyr<sub>358</sub>) are important as a charge relay to carry out reactions without disrupting peroxide binding [32]. The cCAT shares all main conserved features with its human orthologue (Fig. 1). However, there is a considerable difference at the C terminus, where the human enzyme contains the peroxisomal targeting signal (SHL and KANL), which is missing in the *C. fasciculata* orthologue (Fig. 1).

### Heterologously expressed cCAT and hCAT localize to the cytoplasm and do not affect growth in PCF and BSF *T. brucei*

The information about catalase localization in *Crithidia* spp. is somewhat contradictory in the literature.



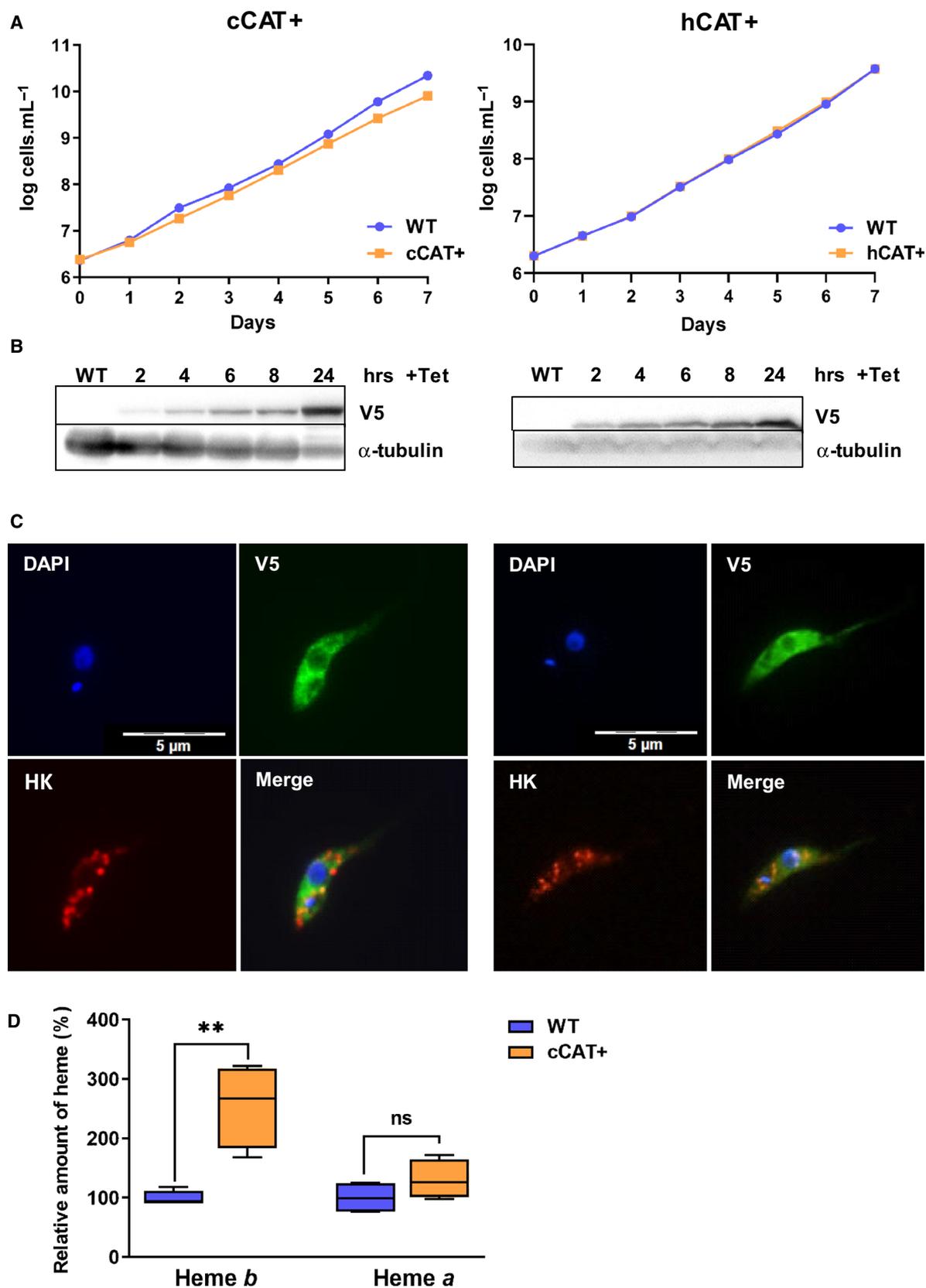
**Fig. 1.** Properties of catalase gene. Sequence alignment of heterologous catalases used in this study. Sequences of *Crithidia fasciculata* (cCAT, TriTrypDB gene ID CFAC1\_250006200) and *Homo sapiens* (hCAT, NCBI gene ID NP\_001743.1) were aligned by SeaView. Rectangular frames show predicted peroxisomal targeting domains. # depicts a conserved His residue at position 75 important for heme binding (numbering is based on the human orthologue). Asterisks indicate residues involved in the active site charge relay.

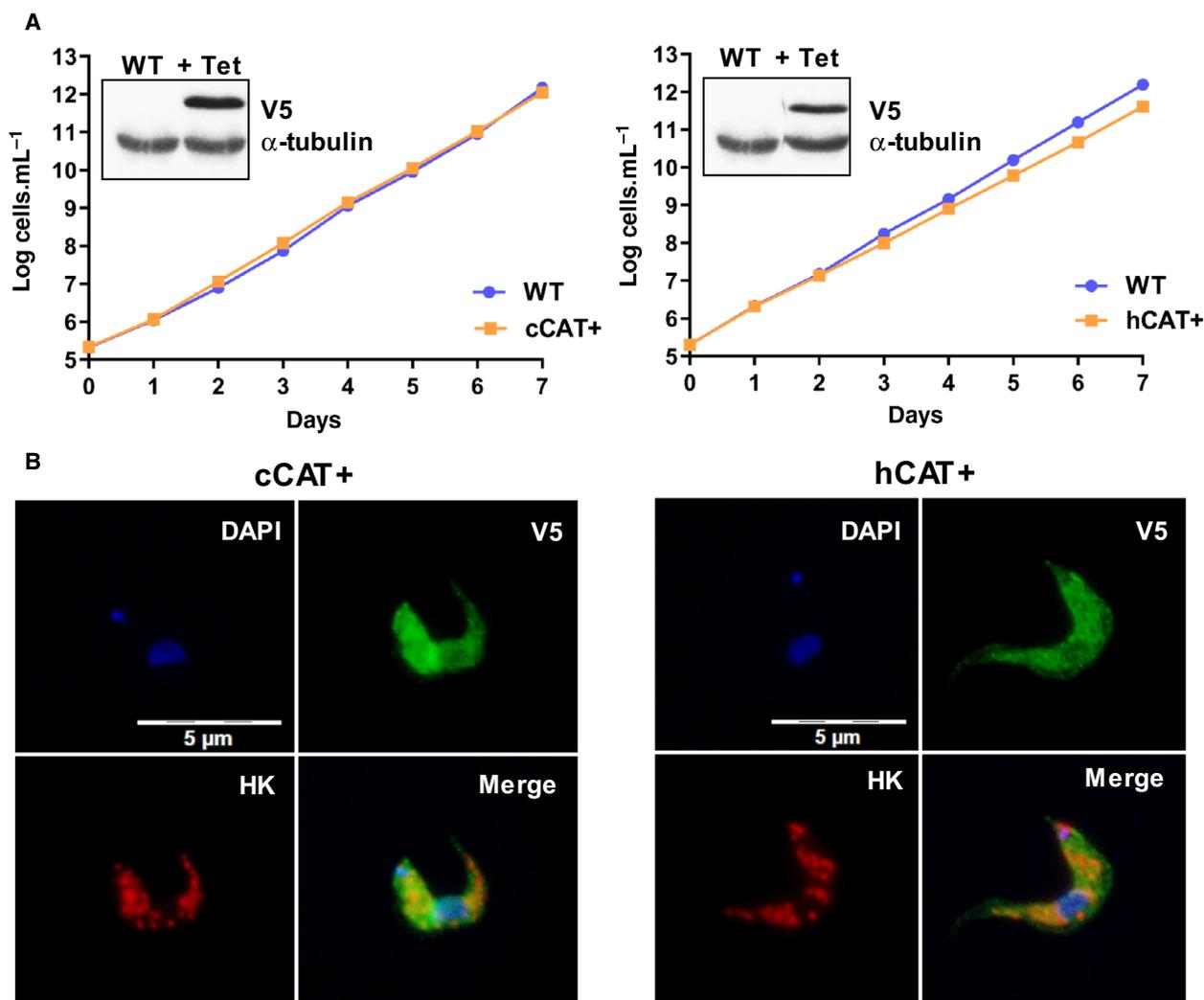
Earlier observations showed that the enzyme is soluble and cytosolic [33], but others detected catalase or at least its activity in the glycosomes [34,35]. It has to be noted that *C. fasciculata* catalase is most likely not localized inside the glycosomes since its sequence does not possess a peroxisomal targeting signal.

To study the distribution of catalase in the cell and its impact on *T. brucei*, we generated a tetracycline (Tet)-inducible PCF cell lines expressing N-terminally V5-tagged catalases from the closely related *C. fasciculata* and phylogenetically more distant human—cCAT and hCAT, respectively. First, we determined the growth rate of the wild-type (WT) and cCAT/hCAT-expressing *T. brucei*. All cell lines

showed a comparable growth, although the cCAT-expressing trypanosomes replicated somewhat slower, starting at day 6 postinduction (Fig. 2A). A gradual increase in catalase was detected after the Tet addition, with the protein being apparent as early as 2 h after induction and stably expressed after 24 h (Fig. 2B). The PCF cell lines were further used to analyze the intracellular localization of cCAT and hCAT by immunofluorescence microscopy. Despite the prediction, both catalases showed uniform cytosolic distribution. The pattern greatly differed from the hexokinase (a glycosomal marker) localization with many discrete vesicles throughout the cell (Fig. 2C).

**Fig. 2.** Expression and localization of different catalases in PCF *Trypanosoma brucei*. Overexpression of the N-terminally V5-tagged cCAT (left) and hCAT (right) in PCF cells. (A) Growth curves of WT (blue line) and cCAT- or hCAT-induced (cCAT+; hCAT+; orange line) cells. A representative growth curve from three biological replicates is shown. (B) Western blot analysis with  $\alpha$ -V5 antibody shows the appearance of the tagged protein cCAT+ or hCAT+ in induced cells (+Tet, in hours) and its absence in WT cells. Alpha-tubulin was used as a loading control. (C) Immunofluorescence assay of cCAT+ and hCAT+ induced cells (+Tet, day 1), which were stained with monoclonal  $\alpha$ -V5 antibody (green),  $\alpha$ -hexokinase (glycosomal marker, red), and DAPI (blue). (D) Content of heme *a* and *b* in WT and cCAT+ (+Tet, day 4) cells. Values in the WT cells were set to 100%. Heme was extracted from  $5 \times 10^8$  cells, separated by HPLC and detected by a diode array detector. Data for three independent biological replicates are shown and presented as mean  $\pm$  SD. Results were analyzed for significant differences using Student's *t*-test (ns,  $P > 0.05$ ; \*\* $P \leq 0.01$ ).





**Fig. 3.** Expression and localization of different catalases in BSF *Trypanosoma brucei*. Overexpression of the N-terminally V5-tagged cCAT (left) and hCAT (right) in BSF cells. (A) Growth curves of WT (blue line) and cCAT- or hCAT-induced (cCAT+; hCAT+; orange line) cells. A representative growth curve from three biological replicates is shown. Insets show western blot analysis with  $\alpha$ -V5 antibody in WT and induced (+Tet, day 1) cells. Alpha-tubulin was used as a loading control. The +Tet samples were run on the same gel, with the same WT control, and the nonadjacent bands have been moved next to each other. (B) Immunofluorescence assay of cCAT+ and hCAT+ induced cells (+Tet, day 1), which were stained with monoclonal  $\alpha$ -V5 antibody (green),  $\alpha$ -hexokinase (glycosomal marker, red), and DAPI (blue).

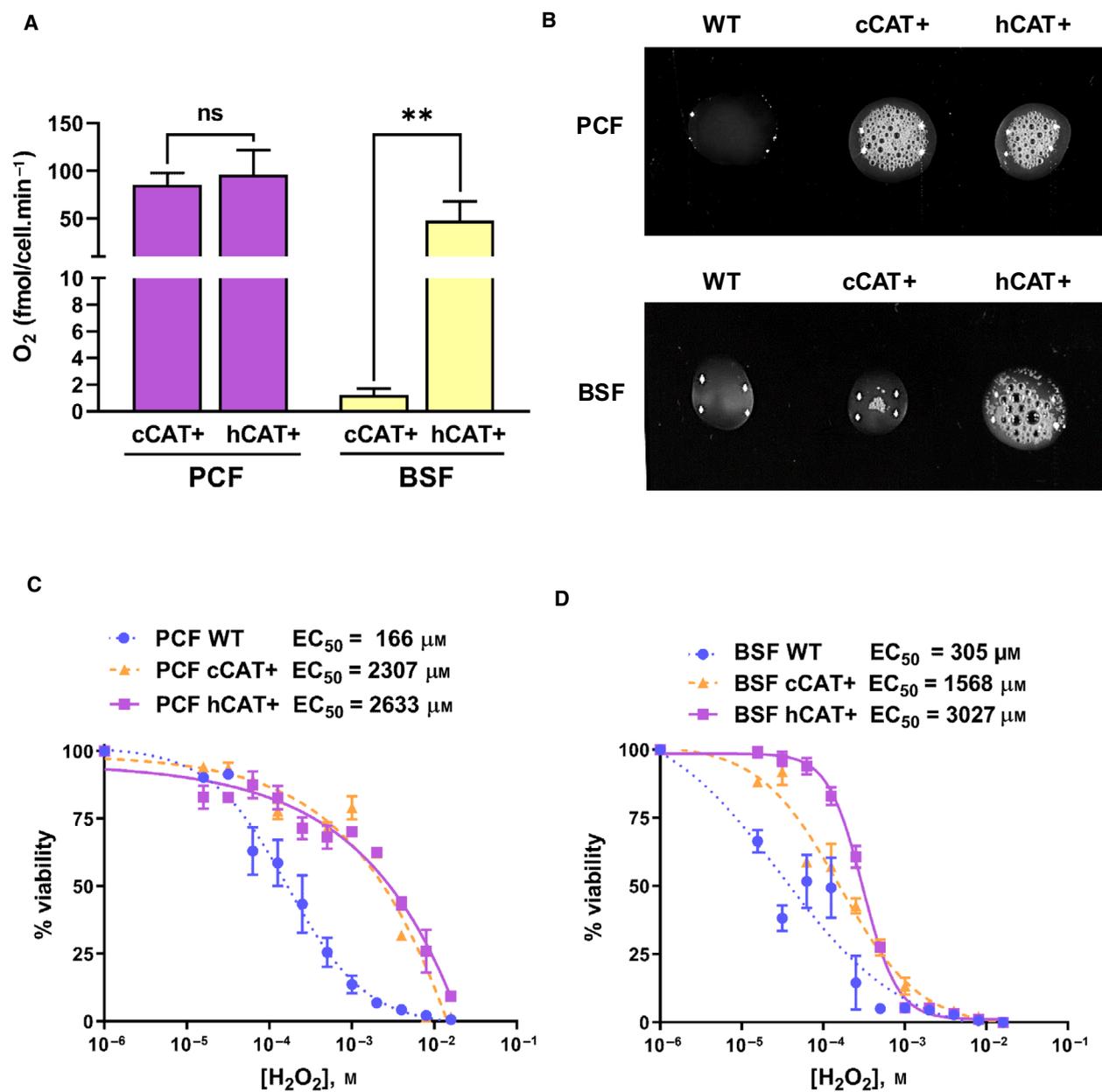
The expression of cCAT in *T. brucei* led to a substantial accumulation of heme *b*. PCF expressing cCAT for 4 days internalized 2.5 times more heme *b* than the WT flagellates (Fig. 2D).

We applied the same strategy, as was used in PCF, and overexpressed cCAT and hCAT also in BSF *T. brucei*. Protein expression was verified by western blots (Fig. 3A; insets). The BSF growth *in vitro* was only marginally affected in the case of hCAT (Fig. 3A). Similarly, as in the PCF, both cCAT and hCAT proteins were confined to the cytosol, where they seem to be uniformly distributed and distinct

from the discrete glycosomal localization of hexokinase (Fig. 3B).

### Expression of catalase in PCF and BSF leads to increased resistance to H<sub>2</sub>O<sub>2</sub>

A unique feature of the catalase reaction (unlike other peroxidases) is the production of molecular oxygen after decomposition of H<sub>2</sub>O<sub>2</sub>. We took advantage of this specific reaction and measured the catalase activity using Oroboros oxygraph, which detects the level of O<sub>2</sub> in the environment. Formation of O<sub>2</sub> was robust in



**Fig. 4.** Different catalases increase resistance to H<sub>2</sub>O<sub>2</sub> stress in PCF and BSF *Trypanosoma brucei*. (A) Measurement of catalase activity using Oroboros oxygraph in PCF cCAT+, PCF hCAT+, BSF cCAT+, and hCAT+ (+Tet, day 1) cells. Data for three independent biological replicates are shown and presented as mean ± SD. Results were analyzed for significant differences using Student's *t*-test (ns, *P* > 0.05; \*\**P* ≤ 0.01). (B) Verification of catalytic activity of catalase by the production of oxygen (bubbles) after the addition of 3% H<sub>2</sub>O<sub>2</sub>. Sensitivity of PCF WT, cCAT+, and hCAT+ (+Tet, day 1) (C) and BSF WT, cCAT+, and hCAT+ (+Tet, day 1) cells (D) to different concentrations of H<sub>2</sub>O<sub>2</sub>. The proliferation of cells and EC<sub>50</sub> values were quantitatively measured by Alamar Blue assay. Data for three independent biological replicates are shown. The data are the mean values ± SD (*n* = 3).

the PCF *T. brucei* expressing cCAT as well as hCAT with comparable values (85 and 95 fmol·min<sup>-1</sup>·cell<sup>-1</sup>, respectively) (Fig. 4A). BSF cells expressing hCAT showed approximately half the activity compared to PCF (48 fmol·min<sup>-1</sup>·cell<sup>-1</sup>), but it was ~40× higher

than that of the cCAT-expressed BSF cells (Fig. 4A). Oxygen is also detectable directly in the cell suspension where it forms macroscopic bubbles after the addition of 3% H<sub>2</sub>O<sub>2</sub> and can be used as an easy readout for the catalase activity. Using this method, we verified

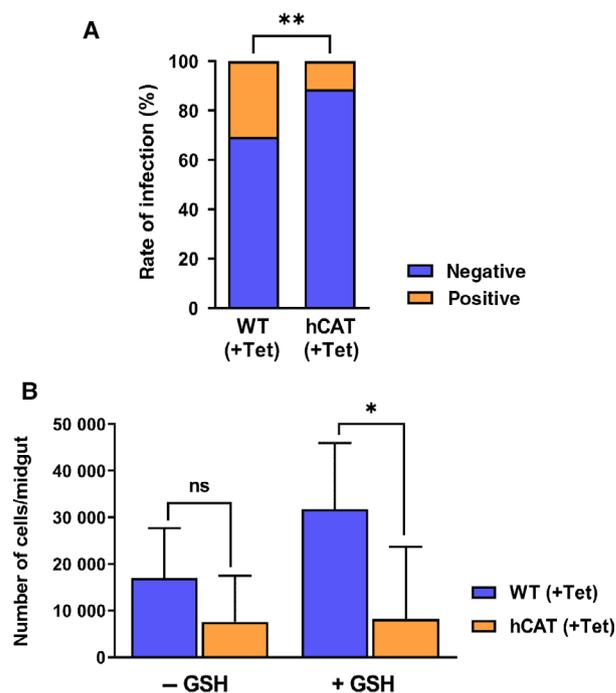
previous results, with the activity of cCAT being similar to that of hCAT in PCF cells. Again, the activity of cCAT in BSF was significantly lower, when compared to hCAT in BSF (Fig. 4B).

Active catalase ensures increased resistance to  $H_2O_2$  as was confirmed in both life cycle stages using Alamar Blue assay. Upon exposure to elevated  $H_2O_2$  concentrations, both PCF CAT-expressing trypanosomes show increased survival rate when compared to the WT (Fig. 4C). Similarly, in BSF hCAT+,  $EC_{50}$  values were  $\sim 10\times$  higher when compared to WT BSF cells. The difference was not as pronounced in BSF hCAT cells with  $\sim 5\times$  higher values in the comparison to WT BSF (Fig. 4D). This indicates that parasites expressing suitable catalase are potentially more resistant to oxidative damage induced by  $H_2O_2$  than their kin lacking the enzyme.

### Catalase decreases the ability of PCF to establish infection in tsetse midgut

After studying the behavior of catalase-expressing PCF cells *in vitro*, we established that both catalases (cCAT and hCAT) have similar activities in culture. However, the situation in BSF was different, with hCAT being more potent than cCAT. Since hCAT was more versatile for both life cycle stages, we decided to use it in PCF and BSF cells for the following *in vivo* experiments. We determined the percentage of established PCF infections in the midgut of tsetse flies that ingested Tet-induced 427 WT or hCAT-expressing PCF. The flies, experimentally infected with catalase-induced PCF, demonstrated a significant decrease in the midgut infection rate compared to those infected with the WT PCF. While the rate of infection was around 30% for the latter cells, the expression of hCAT caused a drop to 11% of positive midgut infection (Fig. 5A).

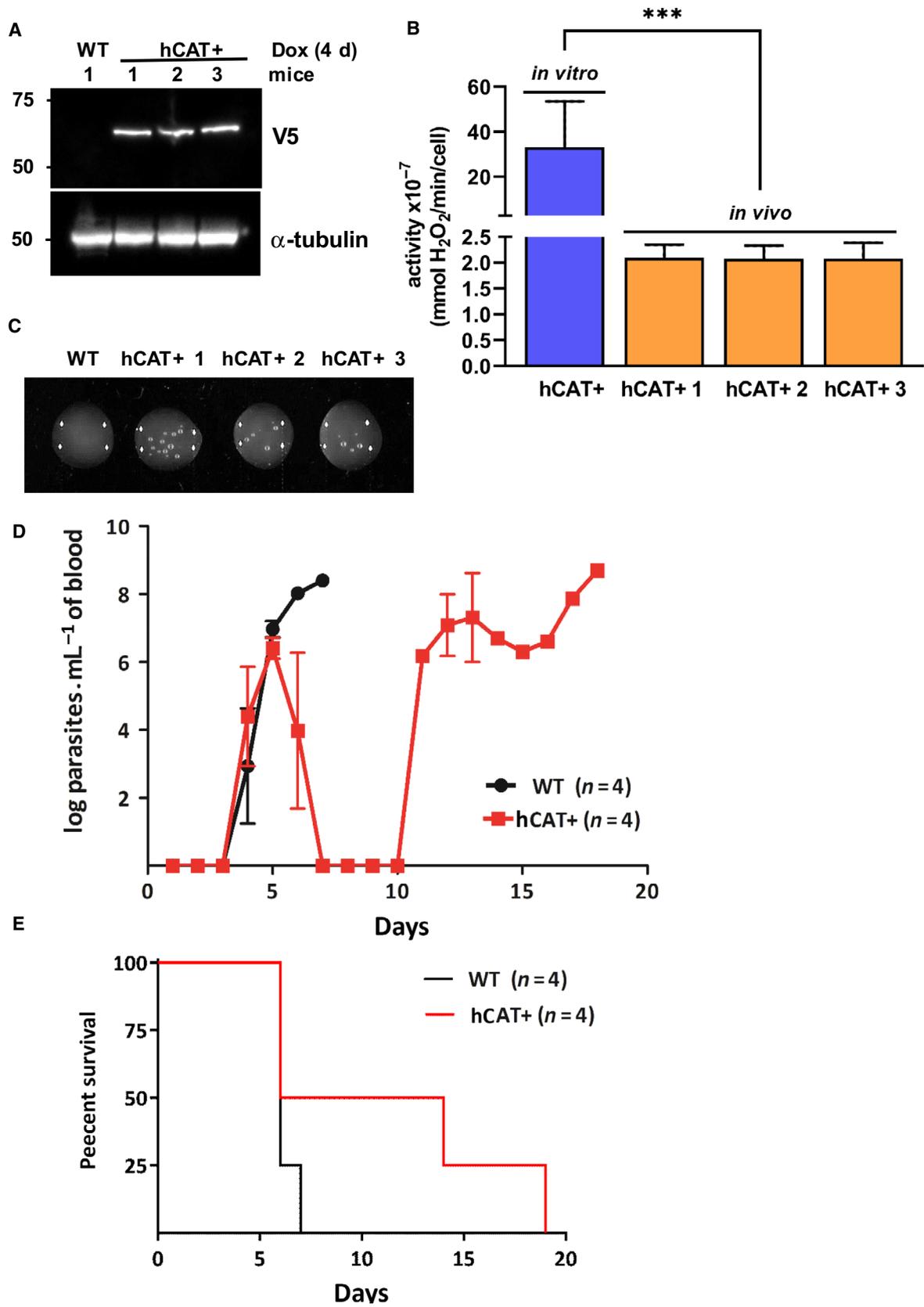
Further, we performed a more detailed evaluation of the infection, where the WT and hCAT PCF Tet-induced cells were quantified by qPCR (Fig. 5B). The data supported our previous observation since the



**Fig. 5.** PCF *Trypanosoma brucei* expressing catalase are compromised in the tsetse flies. (A) Established midgut infection rates at day 17 postinfection of *Glossina morsitans morsitans* flies that were fed on defibrinated horse blood containing  $2 \times 10^6$  cells·mL<sup>-1</sup> of the WT (+Tet;  $n = 85$ ) or hCAT-induced (+Tet;  $n = 104$ ) *T. brucei* PCF cells. (B) The number of parasites during the midgut infection of *G. m. morsitans* with WT (+Tet) or hCAT-induced (+Tet) *T. brucei* PCF cells in the absence (-GSH) or presence (+GSH) of glutathione, respectively. Flies were dissected at day 6 after the first trypanosome-containing blood meal. DNA was subsequently isolated from the total midguts ( $n = 27$  for each group) and quantified by qPCR using 18S rRNA-trypanosome specific primers. Statistical significance was determined by chi-square analysis (ns,  $P > 0.05$ ; \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ). The results are the mean values  $\pm$  SD.

number of hCAT cells in the midgut was lower compared to the WT cells. This difference was significantly more pronounced in the flies when L-glutathione was added to the infective blood meal. The L-glutathione

**Fig. 6.** Human catalase expressed in BSF *Trypanosoma brucei* suppresses parasitemia and prolongs survival in mice. Mice were infected intraperitoneally with 200 WT ( $n = 1$ ) or hCAT+ ( $n = 3$ ) BSF cells pre-induced with  $1 \mu\text{g}\cdot\text{mL}^{-1}$  Tet for 1 day. To maintain induction,  $2 \text{ mg}\cdot\text{mL}^{-1}$  doxycycline (dox) was added to the drinking water 2 days before infection and distributed during the experiment. Parasites were separated from blood using DEAE column and harvested on the 4th day after growth *in vivo*. (A) Western blot analysis with  $\alpha$ -V5 antibody shows the presence of the tagged protein in induced cells (hCAT + dox) and its absence in WT cells (WT + dox). Alpha-tubulin was used as a loading control. (B) Measurement of catalase activity using ferrous method from three mice infected with hCAT + cells and from cultured hCAT+ (+Tet, day 1) cells. The data are the mean values  $\pm$  SD ( $n = 3$ ). Results were analyzed for significant differences using Student's *t*-test (\*\* $P \leq 0.001$ ). (C) Verification of catalytic activity of catalase by the production of oxygen after the addition of 3%  $H_2O_2$  to WT and hCAT+ cells from infected mice, respectively. (D) WT (black line, circles;  $n = 4$ ) and hCAT+ (red line, squares;  $n = 4$ ) cells were counted daily on hemocytometer, and parasitemias were followed for 19 days. The data are the mean values  $\pm$  SD ( $n = 4$ ). (E) Survival was monitored daily and is shown for WT (black line;  $n = 4$ ) and hCAT+ (red line;  $n = 4$ ) cells by a Kaplan–Meier curve.



has been previously demonstrated to facilitate the mid-gut development of trypanosomes [36].

### hCAT reduces parasitemia and extends the survival of infected mice

Firstly, we checked the expression and activity of catalase in BSF isolated from infected mice. The expression of the tagged hCAT was detected only in trypanosomes isolated from mice infected with the hCAT-expressing BSF (Fig. 6A). The enzyme was enzymatically active, although the values were ~10 times lower as compared to those measured in the *in vitro* cultured BSF (Fig. 6B,6). This reduction in enzymatic activity may be attributed to the possible inaccessibility of heme *b* in the blood of mice.

Since, under cultivation conditions, catalase protects *T. brucei* against H<sub>2</sub>O<sub>2</sub>, we hypothesized that its expression might enhance the parasite's survival *in vivo*. To test this, mice were injected intraperitoneally with either the hCAT-expressing or the WT BSF. In the control group, the inoculation of 200 WT BSF was invariably lethal within 7 days. The number of the WTs gradually increased (Fig. 6D; at a detectable level from day 4 onwards), with the highest parasitemia ( $5 \times 10^8$ – $1 \times 10^9$  cells) reached at day 7, causing mortality (Fig. 6E). In contrast, the infection with BSF expressing hCAT resulted in a markedly prolonged host survival (Fig. 6E). Corresponding to the extended survival, the hCAT-expressing cells exhibited a different profile of the parasitemia (Fig. 6D). They proliferated at a normal rate in the first 5 days postinfection; however, at day 7 their numbers dropped dramatically to an undetectable level. The infection bounced back, with increasing level of parasitemia after day 10, which grew to a lethal level by day 19 (Fig. 6D,6).

## Discussion

Reactive oxygen species are continuously generated via the reduction of molecular oxygen to the superoxide anion. If produced *in vivo*, they are generally considered deleterious for eukaryotic cells [37]. However, it is now accepted that at low levels, H<sub>2</sub>O<sub>2</sub> may also act as a critical intermediate in cellular signaling pathways [38]. Cells are generally protected from oxidative damage by intracellular enzymes detoxifying ROS. These include superoxide dismutase catalyzing the production of H<sub>2</sub>O<sub>2</sub> from the more reactive superoxide anion [39], while catalase, glutathione, and thioredoxin peroxidases detoxify H<sub>2</sub>O<sub>2</sub> into water and nonreactive species [40]. Importantly, the antioxidant repertoire of several dioxenous trypanosomatid

flagellates, such as *Trypanosoma* and *Leishmania*, lacks catalase. As we proposed previously, only *Leishmania* spp. have lost catalase secondarily, since representatives of the phylogenetically related to *Leishmania* monoxenous genera from the subfamily Leishmaniinae [41] contain catalase in their genomes [19]. Until recently, it remained unnoticed that the blood-dwelling unicellular and multicellular parasites tend to lose catalase [19]. For instance, the spirochete *Borrelia burgdorferi*, a pathogen infecting hematophagous hosts, lacks not only catalase, but also other peroxidases [42], and expresses a single SOD of the Fe/Mn family that is essential for bacterial virulence [43]. Antioxidants play a significant regulatory role in mid-gut physiology of the hematophagous insects, which are also equipped with their own catalases [10]. An oxygen-rich environment seems to be another important prerequisite for retaining catalase, since the enzyme is absent in the species inhabiting anoxic environments, such as parasitic protists *Giardia*, *Trichomonas*, *Entamoeba*, and *Cryptosporidium* spp. [44]. Similarly, it was shown that catalase from the host red blood cells, which is internalized by *Plasmodium falciparum*, is not essential under low (5%), but is necessary for the parasite protection under high (20%) oxygen concentration [45]. Blood is a microaerophilic environment with only 1.5% of dissolved oxygen, while the rest is bound to hemoglobin [46]. Concentration of H<sub>2</sub>O<sub>2</sub> in the blood is also rather low (1–5 μM), and it is efficiently scavenged by red blood cells [47]. Little is known about oxygen and H<sub>2</sub>O<sub>2</sub> concentrations in the tsetse fly midgut, but the fact that it harbors microaerophilic S-endosymbionts lacking catalase [48] suggests that oxygen concentration there is low. Contrary to the dioxenous species, the monoxenous trypanosomatids such as *Crithidia* and *Leptomonas* spp. undergo a stage in their life cycle when, in the course of transmission between hosts [20], cells are inevitably exposed to an outside environment with high concentration of oxygen. Interestingly, catalase has been shown to function in dormant cells of the microsporidian parasite *Nosema locustae*, indicating that the enzyme may play some functional role in the survival of the spore, which is the only developmental stage outside of the host cell [49].

In order to define the impact of the antioxidant enzyme (catalase) expression on *T. brucei* parasitism, we generated cell lines expressing a heterologous catalase that originated either from the monoxenous *C. fasciculata* or from a human. Our *in vitro* data demonstrated that cells have the ability to express active catalase, protecting them against the oxygen peroxide-induced stress. Under standard *in vitro*

cultivation conditions, the catalase expression does not significantly affect the cell division kinetics. However, after prolonged production of cCAT in PCF, we detected the significant accumulation of heme *b*. Heme can possibly be stored in catalase and used under the heme-limited conditions. It has been shown that after 2 days of heme starvation, only 6% of the catalase activity in *C. fasciculata* was retained, whereas the oxygen consumption and cytochrome-mediated respiration remained unaffected [50]. In *T. brucei*, we recapitulated (to some extent) the phenotype observed in *T. cruzi* after the expression of a bacterial catalase *in vitro* [31]. However, the *in vivo* phenotype was dramatically different. It has been suggested that *T. cruzi* has a buffering system allowing it to maintain a certain level of H<sub>2</sub>O<sub>2</sub>, which is deregulated in the presence of catalase.

Moreover, the expression of catalase contributed to the elevated proliferation of *T. cruzi* in the insect vector but did not significantly increase parasite virulence in the mammalian host [31]. In *T. brucei*, we demonstrated that the induced expression of catalase significantly affected the ability of the insect-dwelling PCF to establish infection and multiply in the tsetse fly midgut. At this point, we can only speculate why catalase represents such a barrier in parasite progression. Tsetse is highly resistant to trypanosome infection, and diverse physiological factors, contributing to this phenotype, have been identified. ROS is just one of them, along with the fly age, nutritional status at the time of exposure to parasites, presence of the antimicrobial peptides, trypanosome-binding lectins, gut-associated EP proteins, and parasite inhibitory peptidoglycan recognition protein LB [51]. In the mammalian host, we have also documented differences between the WT trypanosomes and those, expressing human catalase. We demonstrated that mutants attenuated growth on day 5 postinfection, resulting in a lower first peak of parasites, and, consequently, a substantially increased survival of the infected mice. That is in agreement with the recent observation that protistan parasites rather benefit from the oxidative environment in the mammalian host than suffer from oxidative damage. For example, contesting the notion that *T. cruzi* gets killed by the macrophage-produced ROS [52], it has been recently shown that ROS production promotes *T. cruzi* infection, and mice or macrophages, treated with antioxidants, have reduced parasite burden [53].

Similarly, application of the antioxidant glutathione replenisher, *N*-acetyl-L-cysteine, resulted in the reduced parasitemia in BALB/c mice infected with *Leishmania major* [54]. In contrast, inhibition of

the glutathione synthesis increased *Leishmania* burden [55]. Adding to the picture, APX-overexpressing *Leishmania* did not form lesions [56]. It has been shown that ROS can act as signaling molecules, responsible for the differentiation of virulent *Leishmania* amastigotes [28].

To summarize, the presence of catalase affects the growth potential of trypanosomes *in vivo* in both the insect and the mammalian hosts. Trypanosomes appear to have well-tuned machinery for maintaining redox balance, which enables them to adapt to and survive in conditions imposing oxidative stress. We propose that catalase is not part of this machinery in *Trypanosoma* spp., since its expression results in the reduced ability to proliferate *in vivo*, yet it is needed in the monoxenous flagellates that are likely more exposed to oxygen.

## Materials and methods

### Cultivation and growth curves

*Trypanosoma brucei* 29–13 PCF and 427 BSF-SM (single marker, hereafter called BSF) were routinely cultivated at 27 °C in SDM-79 medium, supplemented with 10% FBS (both from Sigma-Aldrich, St. Louis, MO, USA), or at 37 °C in HMI-9 medium (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% FBS. Cell densities were measured daily using the Z2 Coulter Counter (Beckman Coulter, Brea, CA, USA) and diluted back to densities  $5 \times 10^5$ /mL and  $5 \times 10^6$  cells·mL<sup>-1</sup> for BSF and PCF, respectively.

### Overexpression of catalases

Gene constructs for catalases from *C. fasciculata* (cCAT, TriTrypDB gene ID CFAC1\_250006200) and human (hCAT, NCBI gene ID NP\_001743.1) were prepared using the following primer pairs (added restriction sites are underlined): Cat\_Cfas\_F (5'-gcaggatccccagcaacgagag-3') and Cat\_Cfas\_R (5'-ctgtctagactattagatctccgactc-3'); and Cat\_human\_F (5'-caggatccgctgacagc-3') and Cat\_human\_R (5'-ctgtctagactacagattgccttc-3'). PCR amplicons (annealing temperature 58 °C) from the total genomic DNA of *C. fasciculata* and the plasmid HG12084-G, encoding human catalase ORF (NCBI RefSeq ID NM\_001752.3; Sino Biological, Wayne, NJ, USA), were cloned into the pT7-V5 vector [57] and verified by sequencing. The obtained constructs were linearized by *NotI* and electroporated into both the PCF and BSF cells. The transfected cell lines were selected using 1 µg·mL<sup>-1</sup> of puromycin (Thermo Fisher Scientific). The expression of the tagged protein was induced by addition of 1 µg·mL<sup>-1</sup> of Tet (Sigma-Aldrich) to the medium.

### Western blot analysis and immunofluorescence assay

In order to detect protein expression in the PCF and BSF trypanosomes, lysates from  $5 \times 10^6$  cells were separated on a 12% SDS/PAGE, transferred to a PVDF membrane, and probed with the monoclonal anti-V5 and anti- $\alpha$ -tubulin antibodies (both from Thermo Fisher Scientific) at 1 : 2000 and 1 : 5000 dilutions, respectively. For immunofluorescence analysis, a total of  $10^6$ – $10^7$  cells induced for 24 hours were fixed with 4% paraformaldehyde and settled on microscopic slides. After 10-min incubation at room temperature, they were washed with PBS and permeabilized with 100% ice-cold methanol for 20 min. Cells were incubated with 5% fat-free milk in PBS-Tween (0.05%) for 1 h, followed by incubation with primary (anti-V5 and anti-hexokinase at 1 : 500 and 1 : 200 dilutions, respectively) and secondary Alexa Fluor 488 anti-mouse IgG and Alexa Fluor 555 anti-rabbit IgG antibodies (Thermo Fisher Scientific) at 1 : 1000 dilution for 1 h at room temperature. After the last washing step, cells were stained with DAPI, mounted with an anti-fade reagent (both from Thermo Fisher Scientific), and visualized using a fluorescent microscope Zeiss Axioplan 2 (Carl Zeiss AG, Oberkochen, Germany).

### Activity assays for antioxidant enzymes

The activity of catalase was monitored continuously by following the rate of decomposition of 10 mM  $\text{H}_2\text{O}_2$  using spectrophotometric ferrous oxidation assay. Ammonium ferrous sulfate  $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  oxidation was detected by a linear decrease in absorbance at 560 nm. The amount of decomposed  $\text{H}_2\text{O}_2$  was calculated using the mM extinction coefficient of  $\text{H}_2\text{O}_2$  (0.0436). The enzymatic activity was also measured by respirometry using the Oroboros Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria) as an amount of molecular oxygen produced after addition of  $\text{H}_2\text{O}_2$ . *Trypanosoma brucei* Lister 427 WT PCF and SM BSF were used to measure the experimental background in the respirometry experiment. In short,  $10^6$  of PCF or BSF cells in 2 mL were treated with 20  $\mu\text{L}$  of 882 mM (3%)  $\text{H}_2\text{O}_2$ . The data were analyzed using the OROBOROS DATLAB Software (Oroboros Instruments, Innsbruck, Austria). All measurements and statistics were calculated from three independent biological replicates.

Alternatively,  $5 \times 10^6$  parasites were resuspended in 10  $\mu\text{L}$  PBS and placed on a microscopic slide. The same volume of 3%  $\text{H}_2\text{O}_2$  was added to the cells and mixed, and the formation of oxygen visible as macroscopic bubbles was monitored as a readout for the catalase activity.

### Viability measurement

The cytotoxic effect of  $\text{H}_2\text{O}_2$  was measured by the fluorimetric Alamar Blue assay using the resazurin dye (Thermo

Fisher Scientific), as described previously [58]. The expression of catalase was induced by the addition of Tet 24 h before the assay was performed. Serial twofold dilutions covering a range from 16 to 0.0156 mM  $\text{H}_2\text{O}_2$  were prepared in 96-well plates. WT *T. brucei* and those expressing cCAT were added to the wells to the final concentration of  $2 \times 10^5$  cells·mL<sup>-1</sup>, and the plates were incubated (PCF for 1 h, BSF for 24 h) at standard cultivation conditions. Next, 10  $\mu\text{L}$  of 0.5 mM resazurin in PBS was added into each well and incubation (PCF for 8 h at 27 °C, BSF for 24 h at 37 °C) continued. Fluorescence was read on Tecan Infinite 200 (Tecan, Männedorf, Switzerland) using an excitation and emission wavelengths of 560 and 590 nm, respectively. The data were analyzed by GRAPHPAD PRISM 5.0 software (GraphPad Software Inc., San Diego, CA, USA) using the nonlinear regression and sigmoidal dose-response analysis with the variable slope to obtain EC<sub>50</sub> values. All experiments were performed in triplicate.

### High-performance liquid chromatography

To induce expression of cCAT, late-log-phase PCF and BSF *T. brucei* were treated with Tet for 4 days. Total of  $5 \times 10^8$  parasites was harvested by centrifugation at 1000 g at 4 °C for 10 min and washed three times with PBS on ice. Cells were resuspended in 60  $\mu\text{L}$   $\text{H}_2\text{O}$  and extracted with 400  $\mu\text{L}$  acetone/0.2% HCl, and the supernatant was collected after centrifugation at 1000 g at 4 °C for 5 min. The pellet was resuspended in 200  $\mu\text{L}$  acetone/0.2% HCl and centrifuged as above. Both supernatants were combined, and 150  $\mu\text{L}$  of each sample was immediately injected into a high-performance liquid chromatography machine (Agilent Technologies, Santa Clara, CA, USA) and separated using a reverse-phase column (4  $\mu\text{m}$  particle size, 3.9  $\times$  75 mm) (Waters Inc., Milford, CT, USA) with 0.1 % trifluoroacetic acid and acetonitrile/0.1% trifluoroacetic acid as solvents A and B, respectively. Heme *a* and *b* were eluted with a linear gradient of solvent B (30–100% in 12 min) followed by 100% of B at a flow rate of 0.8 mL·min<sup>-1</sup> at 40 °C. Both hemes were detected by diode array detector Agilent 1200 (Agilent Technologies) and identified by retention time and absorbance spectra according to commercially available standards (Sigma-Aldrich).

### Fly infections

Freshly emerged *Glossina morsitans morsitans* male tsetse flies were used in the infection experiments. Within 24–48 h after emergence, flies were fed their first blood meal through *in vitro* membrane feeding on a mixture of PBS-washed horse red blood cells (E&O Laboratories, Burnhouse, UK) with PCF trypanosomes in antibiotic-free SDM79 culture medium at a final concentration of  $2 \times 10^6$  cells·mL<sup>-1</sup>. Overexpression of cCAT and hCAT

was induced 1 day before the infection by the addition of  $1 \mu\text{g}\cdot\text{mL}^{-1}$  Tet to the medium. Only fully engorged flies were selected and subsequently maintained for 14 days by feeding three times per week on sterile defibrinated horse blood. Next, the midguts were dissected at day 17 (3 days after the last blood meal) and examined by phase-contrast microscopy for the presence of an established PCF infection. Statistical significance was determined by chi-square analysis. In another experiment, flies were fed their first blood meal with the Tet-induced WT and hCAT-expressing PCF, in the absence or presence of 10 mM reduced L-glutathione to enhance the establishment of trypanosomes in the tsetse midgut [36]. Then, flies received an additional blood meal containing  $1 \mu\text{g}\cdot\text{mL}^{-1}$  Tet and were dissected at day 6 after the first trypanosome-containing blood meal. DNA was isolated from the total midguts using the EZNA Tissue DNA Kit (Omega Bio-tek, Norcross, GA, USA) and used to quantify the parasites by qPCR analysis using 18S rRNA-trypanosome specific primers (18S-F: 5'-cgccaagctaatacatgaaccaa-3' and 18S-R: 5'-taatttcattcctgctg-gacg-3'). DNA extracts from a dilution series of PCF, spiked with uninfected tsetse fly midguts, were used to set up the standard curve.

### Mouse infections

Four- to six-week-old BALB/c mice were injected i.p. with 200 parasites. Mice were watered with  $2 \text{ mg}\cdot\text{mL}^{-1}$  doxycycline (Sigma-Aldrich) in 5% sucrose beginning 2 days before the infection. Overexpression of hCAT was induced 1 day before the infection by addition of  $1 \mu\text{g}\cdot\text{mL}^{-1}$  Tet. BALB/c mice were infected with WT BSF or hCAT-expressing BSF parasites, and the infection was followed for 19 days. Parasitemia was measured daily by diluting tail snip blood into TryPCFix buffer (3.7% formaldehyde,  $1 \times$  SSC buffer) and manual counting of the parasites in a Neubauer hemocytometer. Out of five, one animal from each group never manifested parasitemia throughout the experiment and was, therefore, excluded from the analysis. Mice were euthanized on day 20 for collection of parasites which were separated from the red blood cells on a diethylaminoethyl (DEAE) cellulose column using a standard protocol. The collected parasites were washed once with PBS and subsequently used for downstream experiments.

### Ethics statement

The research was conducted under ethical protocols approved by the Institute of Parasitology, Biology Centre, and Central Commission for Animal Welfare, Czech Republic (protocol no. 28/2016). All experimental procedures complied with the law of the Czech Republic (Act No. 246/1992 Coll., On the protection of animals against cruelty).

### Statistical analysis

Statistical analyses were performed using GRAPHPAD PRISM 8.0 (GraphPad Software Inc.). Data are presented as mean  $\pm$  SD. Results were analyzed for significant differences using either chi-square test or Student's *t*-test (ns,  $P > 0.05$ ; \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ).

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### Conflict of interest

The authors declare no conflict of interest.

### Author contributions

JL and VY financed the research and supervised the project. EH, DF, NK, BK, and JVDA carried out the experiments and analyzed the data. EH, NK, JL, and VY wrote the paper.

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