SUPPLEMENTARY APPENDIX

A role for hepcidin in the anemia caused by *Trypanosoma brucei* infection

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Methods

Mice, Parasites, Infections and Sampling

All animals were housed in the animal facility of the Instituto de Investigação e Inovação em Saúde (i3S), kept in individually ventilated cages with high efficiency particulate air (HEPA) filters and were fed *ad libitum* with sterilized food and water. All experiments were carried out in accordance with the IBMC.INEB Animal Ethics Committees and the Portuguese National Authorities for Animal Health guidelines according to the statements on the directive 2010/63/EU of the European Parliament and Council. Mice were infected by intraperitoneal injection with 100 µl containing 10^5 blood parasites harvested at the first peak of parasitemia from a donor mouse. Control animals were similarly injected with the same volume of PBS. Parasitemia was assessed with a hemocytometer, using blood collected by tail vein puncture, diluted in equal parts with Ethylenediaminetetraacetic acid (EDTA) and further diluted to the appropriate density in 0.85% ammonium chloride to enable erythrocyte lysis. The detection limit achieved was $1x10^5$ parasites/ml. Parasite loads were assessed using the IVIS Lumina. *T. b. brucei* GVR35-infected mice were anesthetized with 2.5% isoflurane (O_2 flow of 1 L/min). D-luciferin (100 mg/Kg, Perkin Elmer, Waltham MA, USA) was administered subcutaneously in the neck five minutes prior to image acquisition. Mice were then transferred to the stage of an intensified charge-coupled device photon-counting video camera box where anesthesia was maintained with 2.5% isoflurane (O_2 flow of 0.3 L/min). Exposure to isoflurane was standardized among groups and throughout all time points and signal acquisition was controlled by the Living Image software (Perkin Elmer). The detection of the bioluminescence signal by the system resulted in the generation of signal maps automatically superimposed to the grey-scale photograph of the mice. The regions of interest (ROI) encompassing most of the ventral view of the animal body, the head, the thorax and the abdomen were manually defined. The quantifications were performed using the Living Image software (Perkin Elmer). The total flux (photons/second)

and average radiance (photons/second/cm^2/steradian) within these ROIs were automatically calculated. The percentage of bioluminescence signal in the defined regions was calculated by dividing the total flux of the respective ROI by the total flux of the ventral animal body ROI. After imaging, blood was collected while the animals were still anesthetized, after which they were sacrificed by cervical dislocation. For each experimental animal, liver, spleen, kidney and bone marrow from femur were collected, weighted and immediately frozen in liquid nitrogen and stored at -80ºC until further use.

Hematological parameters, serum parameters and tissue iron content. For determination of hematological parameters (red blood cells, reticulocytes, hemoglobin, hematocrit, mean corpuscular volume), 100 µl of blood were used in 1:1 dilutions with EDTA (1:10 diluted in sterile PBS) (BD Biosciences, San Jose CA, USA). For determination of serum parameters (serum iron, serum ferritin, transferrin saturation, unsaturated iron binding capacity, total iron binding capacity), non-heparinized blood was transferred into 1.5 ml microcentrifuge tubes, allowed to clot for 4 h at 4ºC, and centrifuged at 16000×g until a clear serum was obtained. For non-heme iron measurements, briefly, tissue samples with an average weight of 50 mg were placed in iron-free Teflon vessels (ACV-Advanced Composite Vessel, CEM Corporation, Matthews NC, USA) and dried in a microwave oven (MDS 2000, CEM Corporation). Subsequently, dry tissue weights were determined and samples digested in an acid mixture (30% hydrochloric acid and 10% trichloroacetic acid) for 20 h at 65ºC. After digestion, a chromogen reagent (5 volumes of deionised water, 5 volumes of saturated sodium acetate and 1 volume of 0.1% bathophenanthroline sulfonate/1% thioglycollic acid) was added to the samples in order to react with iron and obtain a colored product that was measured spectrophotometrically at 535 nm. The *extinction coefficient* for bathophenanthroline is 22.14 $mM^{-1}cm^{-1}$.

Cytokine profile analysis. The BD Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, San Jose CA, USA) allows the simultaneous quantification of Interleukin(IL)-6, IL-10, Tumour Necrosis Factor(TNF)-alpha, Interferon(IFN)-gamma and Monocyte Chemoattractant Protein(MCP) 1. Briefly, serum samples were diluted 1:5 v/v in Assay Diluent, and plated in 96-well plates with mixed Capture Beads and PE Detection Reagent. The mixture was incubated for 2 h at RT in the dark, after which wash buffer was added prior to centrifugation for 1 min at 200×g. The supernatant was discarded and the beads ressuspended in wash buffer. Samples were run in a BD FACSCanto II Flow Cytometer and results were analysed with the FCAP Array Software (BD Biosciences).

Flow cytometry. Bone marrow cells were isolated by flushing long bones in DMEM supplemented with 2% heat-inactivated fetal bovine serum, L-Glutamine and PenStrep (all from Thermo Fisher Scientific, Waltham MA, USA). Cells were counted using the trypan blue exclusion assay. A million bone marrow cells were plated and stained with anti-CD3e (17A2), anti-CD19 (6D5), anti-TER119, anti-CD71 (RI7217), anti-CD11c (N418), and anti-CD11b (M1/70) antibodies for 30 min on ice. All antibodies were obtained from BioLegend (San Diego CA, USA). Cells were then washed and fixed with 2% PFA for 10 min at room temperature. Cells were run in a BD FACSCanto II Flow Cytometer (BD Biosciences) and data was analyzed with FlowJo software (FlowJo LCC, Ashland OR, USA). Erythroblast populations were gated based on the expression of the surface markers TER119 and transferrin receptor 1 (CD71) into pro-erythroblasts, basophilic and polychromatic erythroblasts.

Analysis of gene expression by quantitative RT-PCR.

Gene expression was analysed using a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules CA, USA). Genes analyzed include genes involved in iron homeostasis and

regulation (hepcidin, *Hamp1*; erythroferrone, *Erfe*; twisted gastrulation BMP signaling modulator 1, *Twsg1*; transferrin receptor 1, *Tfr1*; growth differentiation factor 15, *Gdf15*), hematopoiesis (erythropoietin, *Epo*; erythropoietin receptor, *Epor*; hemoglobin, *Hbb*), iron storage (ferritin H, *Fth*), iron export (ferroportin, *Fpn1*) and iron transport (transferrin, *Tf*). One µl of each cDNA sample was added to a reaction mix containing 10 µl iQ SYBR Green Supermix (Bio-Rad), 7 µl of $_{dd}H_20$ and 250 nM of each primer (Supplementary Table 1), making a total volume of 20 µl per reaction. A non-template control was included for each set of primers. The cycling profile was as follows: 95ºC for 3.5 min, 40 cycles of 95ºC for 20 s and 59ºC for 20 s. Samples were prepared in duplicates, a melting curve was generated for every PCR product to confirm the specificity of the assays and a dilution series was prepared to check the efficiency of the reactions. Messenger RNA expression was normalized to Hypoxanthine Guanine Phosphoribosyltranferase 1 (*Hprt*) mRNA.

Analysis of ferroportin protein levels by Western blot. *Protein extraction.* Tissues were homogeneized with a tissue grinder in RIPA buffer (3 µl/mg of tissue), supplemented with 10 µl PMSF 100 mM, 10 µl protease inhibitors and 1 µl DTT 1M per ml of buffer, and placed on ice for 20 min. Samples were then centrifuged for 20 min at 14000×g at 4ºC, and supernatants collected. Protein concentration was determined with the Bio-rad DC Protein Assay (Bio-Rad, Hercules CA, USA). *Western blot.* Protein extracts were prepared in Laemmli buffer (Bio-rad). Equivalent amounts of protein (30 µg) were separated by electrophoresis in 12% SDS-polyacrylamide gels (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane for 90 min at 100 V. After blocking the membrane with 5% BSA in TBST (50 mM Tris-HCl, pH 8; 154 mM NaCl; 0.1% Tween 20) overnight at 4ºC, membranes were incubated with the primary antibody following the manufacturer's instructions: rabbit anti-FPN (Ferroportin/SLC40A1 Antibody from Novus, Littletown, CO, USA catalog #NPB1-21502), (1:1000) 1 h RT, rabbit anti-GAPDH

(Abcam, Cambridge, UK, catalog #EPR16891), (1:1000) 1 h RT. Membranes were washed and incubated with the secondary anti-rabbit (1:10000) in 1% BSA in TBST. Membranes were then incubated with the Luminata Crescendo Western HRP substrate, imaged with ChemiDoc imaging system (Bio-Rad) and analysed in ImageLab software (Bio-Rad).

Histological analysis. Sections of liver and spleen were fixed in 10% neutral buffered formalin (Bio-Optica, Milan, Italy) at 4°C for 8 h. After dehydration, sections were embedded in paraffin, sectioned at 3 μm, and stained with Perl's Prussian Blue for ferric iron visualization. Control sections were also stained with hematoxylin and eosin (H&E).

Results

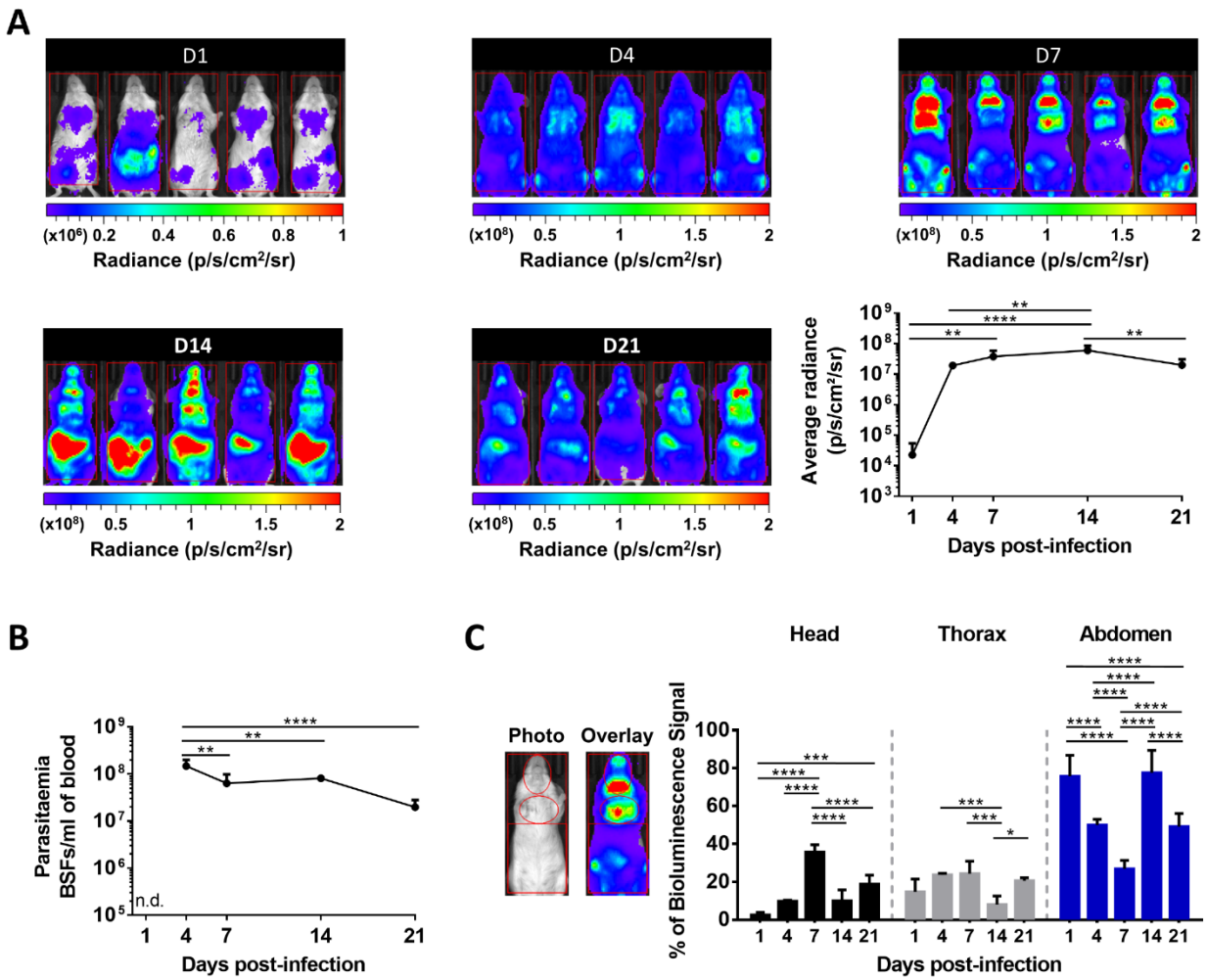
T.b. brucei infection in mice leads to macrocytic anemia, decreased erythropoietic activity and iron redistribution

The bioluminescence signal was detectable at day 1 and when parasites were still undetectable by microscopic analysis of peripheral blood (Supplementary Figure 1A,B). Once parasite burden increased, bioluminescence was detected, as expected, along the entire body of the animal (day 4). Hotspot signal appearing in the head region and indicative of infection of the central nervous system was visible at day 7 (Supplementary Figure 1A). Parasitemias reached a peak at day 4 and after a slight decrease on day 7 were maintained at relatively steady values close to 10^8 parasites/ml of blood (Supplementary Figure 1B). As expected, both methodologies show comparable progression of parasite burden, with the exception of day 1, when parasites were still undetectable by microscopic analysis of peripheral blood. To study the dynamics of parasite distribution and dissemination, the images obtained for each animal were segmented into several areas corresponding to the whole body, head, thoracic region and abdomen (Supplementary Figure 1C). This analysis showed a progressive increase on the percentage of the bioluminescent signal in the head

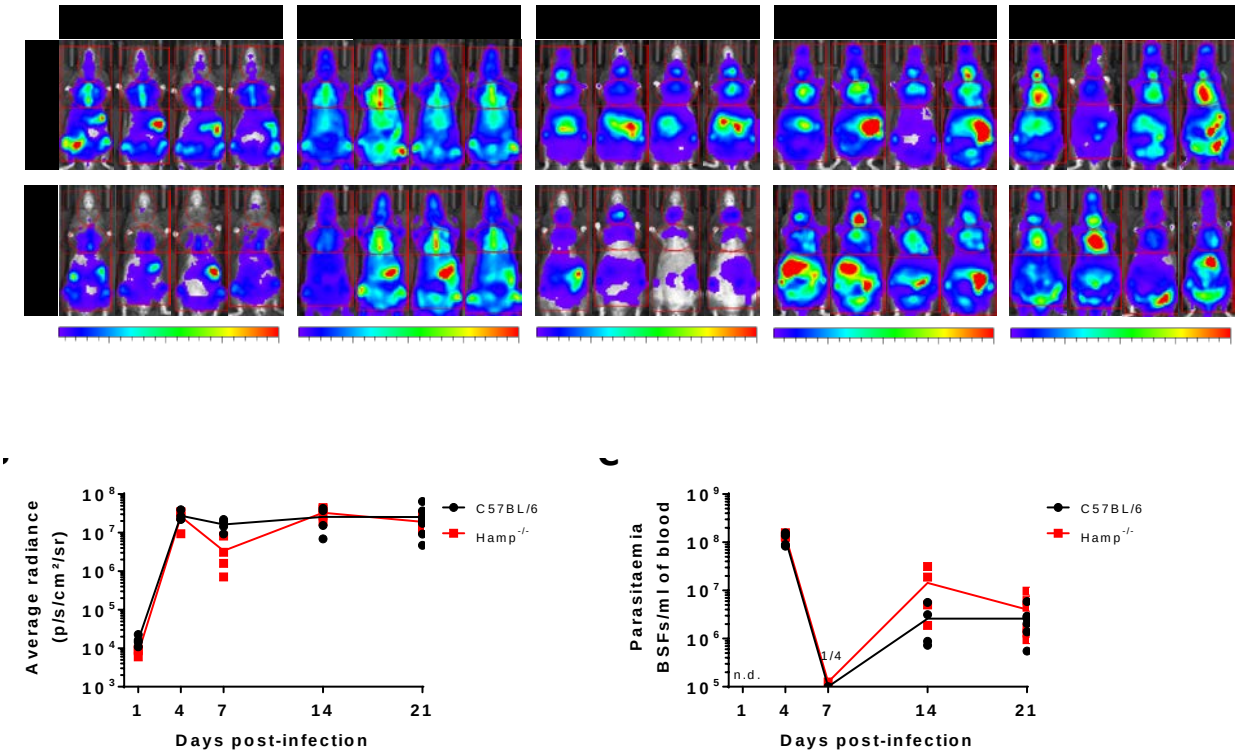
of the animals peaking at day 7 (Supplementary Figure 1C). From day 14, most of the bioluminescence signal detected corresponds to the abdominal region, which might be due to colonization of tissues such as the adipose tissue[31].

Supplementary Table 1. Primers used for gene expression analysis.

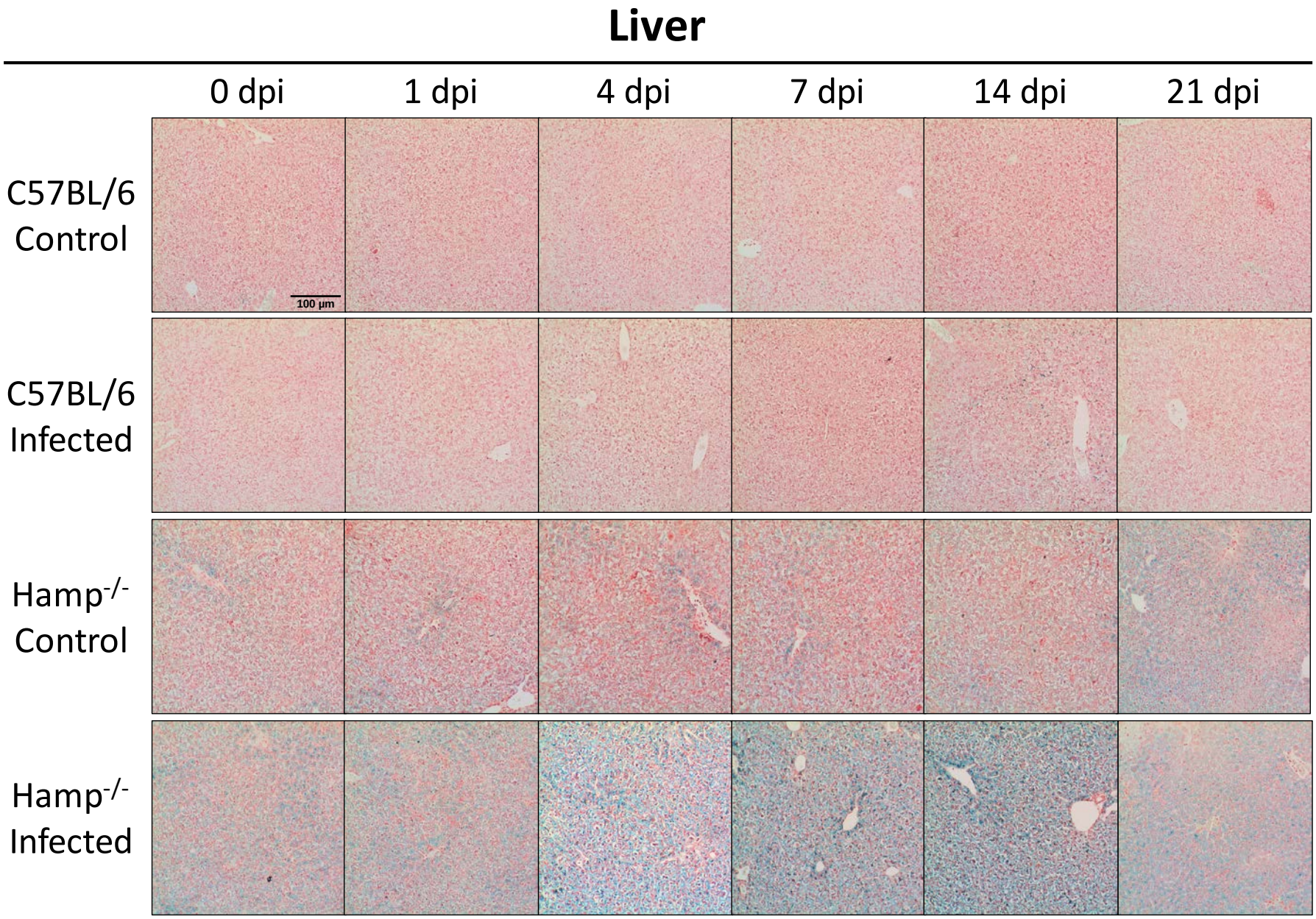
	Forward (5'→3')	Reverse (5'→3')
Hprt	GGTGGAGATGATCTCTCAAC	TCATTATAGTCAAGGGCATATCC
Epo	CCACCCTGCTGCTTTTACTC	TCAGTCTGGGACCTTCTGCA
Epor	TCCTCCTGCTCATCTGCTTT	AGTAGGGGCCATCGGATAAG
Erfe	ACTACCTGCCCGAAGTTGAG	TTGTGCTGGCAGAGAGACTG
Fth	GCTGAATGCAATGGAGTGTGCA	GGCACCCATCTTGCGTAAGTTG
Fpn1	CGCAGAGGATGACGGACACATTC	TTGGTGACTGGGTGGATAAGAATGC
Gdf15	CCGAGAGGACTCGAACTCAG	TTCAGGGGCCTAGTGATGTC
Hamp1	CCTATCTCCATCAACAGATG	AACAGATACCACACTGGGAA
Hbb	ATGGCCTGAATCACTTGGAC	GAGCAGGAAAGGGGGTTTAG
Tf	ACCTGGAACAACCTGAAAGG	GGCCAATACACAGGTCACAG
Tfr1	GCAGCATTGGTCAAAACATGG	GCTTTGGGCATTTGCAACCC
Twsg1	TGACGTTCCTGCTGTGTCTC	ATTCCGAGGGTTGCACATAC



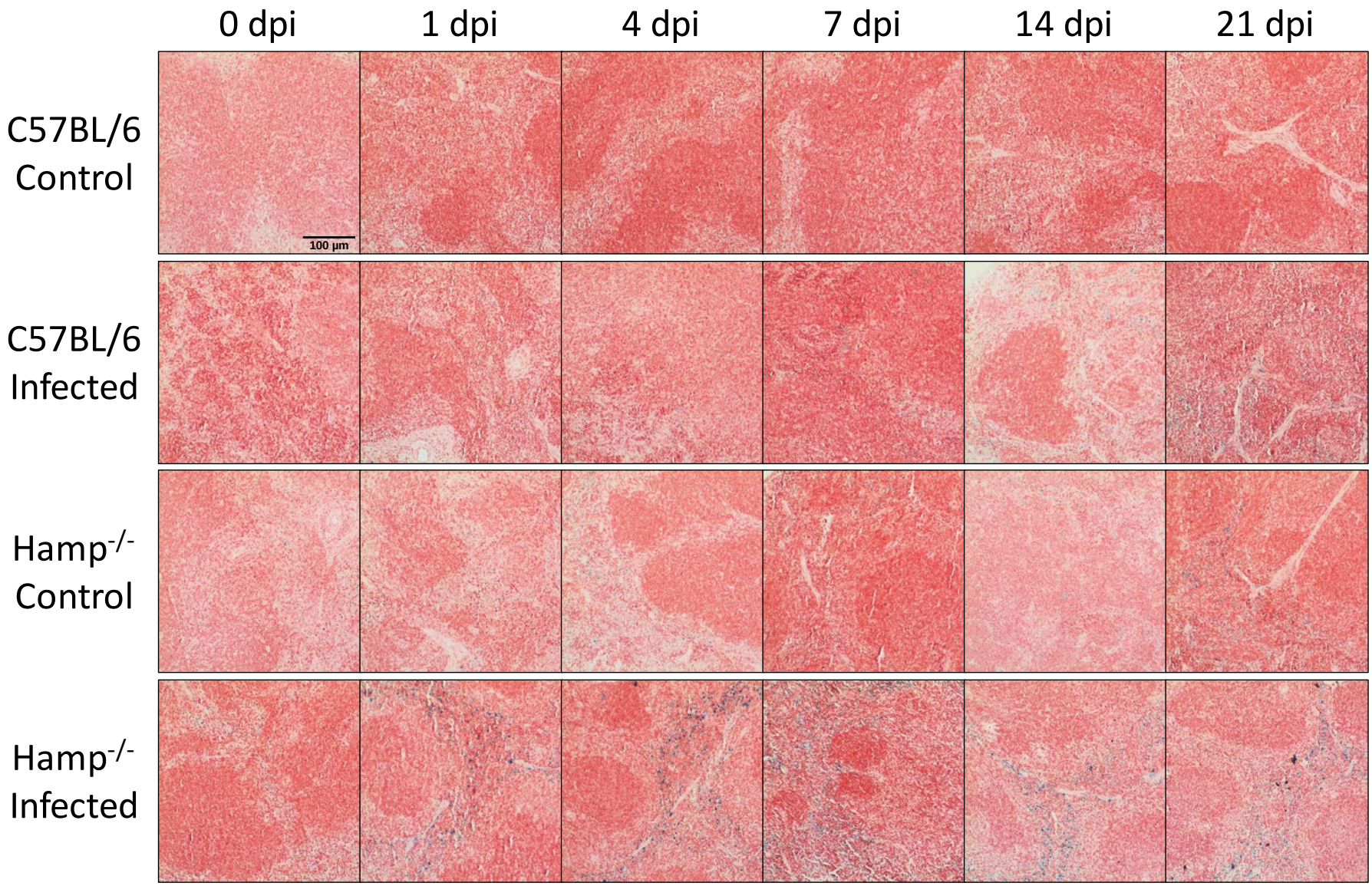
Supplementary Figure 1 - Parasite burdens during the course of infection with *T. b. brucei* in BALB/c mice. (A) Female BALB/c mice infected with 10^5 luciferase-expressing *T. b. brucei* BSFs by intraperitoneal injection. Whole-mouse bioluminescence imaging was performed 1, 4, 7, 14, and 21 days post-infection. Images of infected animals are shown and the bioluminescence expressed in average radiance (photons per seconds per square centimeter per steradian [p/sec/cm2/sr]) was quantified. (B) Parasitemia of infected animals. The detection limit was 1×10^5 parasites/ml. n.d. not detected. (C) Percentage of bioluminescence signal throughout the infectious process in different regions of interest (ROIs), namely head, thorax and abdomen. Representative images containing the defined ROIs are shown. Values are represented as means + standard deviation (n=5). Differences between days post-infection were considered significant at *p<0.05, ***p<0.001 and p<0.0001.



Supplementary Figure 2 - Parasite distribution during the course of infection with *T. b. brucei* in C57BL/6 and Hamp$^{-/-}$ mice. (A) Female C57BL/6 and Hamp$^{-/-}$ mice infected with 10^5 luciferase-expressing *T.b. brucei* BSFs by intraperitoneal injection. Whole-mouse bioluminescence imaging was performed 1, 4, 7, 14, and 21 days post-infection. (B) Quantification of bioluminescence expressed in average radiance (photons per seconds per square centimeter per steradian [p/sec/cm2/sr]). (C) Parasitemia of infected animals. On day 7 post-infection only one animal in each group had detectable parasites in the blood. The detection limit was 1×10^5 parasites/ml. n.d. not detected.


Liver
0 dpi
1 dpi
4 dpi
7 dpi
14 dpi
21 dpi
C57BL/6 Control
C57BL/6 Infected
Hamp-/- Control
Hamp-/- Infected
100 µm

Spleen


0 dpi
1 dpi
4 dpi
7 dpi
14 dpi
21 dpi
C57BL/6 Control
C57BL/6 Infected
Hamp-/- Control
Hamp-/- Infected
100 µm

Supplementary Figure 3 – Tissue iron content evaluated by histological analysis, with Perl's method for iron staining. Iron content was evaluated in the liver and spleen of uninfected and infected C57BL6 and *Hamp*$^{-/-}$ mice, at 0, 1, 4, 7, 14, and 21 days post infection (dpi). Higher basal levels could be observed in both the liver and spleen of *Hamp*$^{-/-}$ mice, as well as a continuous deposition in both of infected C57BL/6 and *Hamp*$^{-/-}$ mice, but clearly more noticeable in the later. Blue staining denotes accumulation of ferric iron.