Heme exporter FLVCR regulates mitochondrial biogenesis and redox balance in the hematophagous insect *Rhodnius prolixus*

Ana Beatriz Walter-Nuno[1,2], Mabel Taracena-Agarwal [1,2,*], Matheus P. Oliveira[1**], Marcus F. Oliveira[1,2], Pedro L. Oliveira[1,2] e Gabriela O. Paiva-Silva[1,2]

1-Programa de Biologia Molecular e Biotecnologia, Instituto de Bioquímica Médica Leopoldo de Meis, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, 2- Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular (INCT-EM), Rio de Janeiro, Brazil,

present addresses: * Entomology Department. College of Agriculture and Life Sciences, Cornell University. 129 Garden Ave, 5134. Ithaca, NY, 14850; **Capacity Bio, Inc., Magnify at CNSI, CA, USA, 90095.

For correspondence: Gabriela O. Paiva-Silva (gosilva@bioqmed.ufrj.br) and Ana Beatriz Walter-Nuno (abnuno@bioqmed.ufrj.br)

Running title: FLVCR interferes in mitochondrial biogenesis and physiology

Keywords: heme, mitochondrial metabolism, mitochondria biogenesis, redox imbalance, reactive oxygen species (ROS), antioxidant, FLVCR, heme oxygenase, *Rhodnius prolixus.*

Abstract (250 words)

Heme is a prosthetic group of proteins involved in vital physiological processes in aerobic organisms. It participates in redox reactions crucial for cell metabolism due to the variable oxidation state of its central iron atom. However, excessive heme can be cytotoxic due to its prooxidant property. Therefore, the control of intracellular heme levels ensures the survival of organisms, especially those that deal with high concentrations of heme during their lives, such as hematophagous insects. The Feline Leukemia Virus C Receptor (FLVCR) is a membrane protein responsible for heme transport in mammalian cells. In our study, we found that RpFLVCR serves as a heme exporter in the midgut of the hematophagous insect *Rhodnius prolixus*, a vector for Chagas' disease. Silencing RpFLVCR decreased hemolymphatic heme levels and increased intracellular dicysteinyl-Biliverdin, a product of *R. prolixus* heme degradation, indicating heme retention inside midgut cells. FLVCR silencing led to increased expression of Heme Oxygenase (HO), Ferritin, and Mitoferrin mRNAs while downregulating iron importers Malvolio 1 and 2. In contrast, HO gene silencing increased FLVCR and Malvolios expression and downregulated ferritin, revealing a crosstalk between heme degradation/export and iron transport/storage pathways. Furthermore, RpFLVCR silencing strongly increased oxidant production and lipid peroxidation, reduced cytochrome c oxidase activity and activated mitochondrial biogenesis, effects not observed in RpHO-silenced insects. These data supports the FLVCR function as a heme exporter, playing a pivotal role in the heme / iron metabolism and maintenance of redox balance, especially in an organism adapted to face extremely high concentrations of heme.

Introduction

Heme, an iron-protoporphyrin IX complex, plays a crucial role in the aerobic metabolism of most organisms. It is the prosthetic group of hemeproteins that are involved in a vast array of cellular processes such as antioxidant protection and xenobiotics detoxification. Due to is ability of binding diatomic gases, heme is involved in oxygen transport and NO and CO-dependent signaling pathways. Moreover, it is directly involved in most oxidative reactions required for electron transfer and energy transduction in the mitochondria (1–3).

In order to safeguard cellular integrity and its function, it is essential to uphold appropriate levels of intracellular heme, as an excess heme can be cytotoxic either by directly promoting oxidative damage to biomolecules (4–6) or by altering the membranes permeability (7). In most aerobic organisms, heme homeostasis is maintained by orchestrating the heme synthesis, degradation, and transport between and within cells, according to the supply and demand of this metallocofactor (8–12).

The control of heme homeostasis is particularly critical for hematophagous insects, including vectors of human diseases, which deal with large amounts of this molecule released during the digestion of the host's blood (13). In fact, a variety of mechanisms to counteract the harmful effects of ‘free heme and generated reactive oxygen species (ROS) has been evolved in these animals (13). Some of them represent obvious adaptations of organisms that face an overload of heme, such as the formation of insoluble heme aggregates (hemozoin) (14,15), the sequestration by heme-binding proteins associated to extracellular matrixes (16) and unique heme enzymatic degradation pathways that result in the production of water-soluble modified biliverdins (17,18). While the biochemical aspects of heme synthesis and degradation have been already described in some of these blood-sucking arthropods (17–20), the precise mechanisms involved in transmembrane heme transport remain largely unexplored. In ticks, an ABCB10 homolog has been incriminated in heme transport (21). In contrast, in *Aedes aegypti*, exposing cells and tissues to low or high heme did not come with positive identification of heme transporters (22).

In mammals, the cellular transmembrane heme export is attributed to the Feline Leukemia Virus Subgroup C Receptor 1 (FLVCR1). FLVCR1 was originally identified as the cell surface receptor for the feline leukemia virus subgroup C (23,24), but a role in heme metabolism soon became apparent (25). Flvcr1a is one of the two isoforms codified by the Flvcr1 gene. It is a member of the SLC49 family of the major facilitator superfamily of secondary transporters, capable of transporting small solutes in response to chemiosmotic ion gradients (26,27). Flvcr1a mRNA is widely expressed in various mammalian organs, such as bone marrow, liver, duodenum, lung, kidney, spleen, brain and placenta (28–30). The role of FLVCR1a (here in mentioned as FLVCR) as a heme exporter has been described in different cell types, including erythroid cells, macrophages, hepatocytes, enterocytes and endothelial cells (25,28,31–33). Its contribution to the maintenance of heme homeostasis comes across as particularly critical in erythroid cells, where heme is abundantly produced during erythropoiesis (34). In these cells, FLVCR ensures the efficient transport of the excess of unbound heme molecules, possibly preventing their toxic accumulation (28). However, the role of cellular heme efflux ascribed to FLVCR as an antioxidant mechanism is still controversial (35). FLVCR1-related protein named FLVCR2 has been proposed to be a heme importer in mammals (36) but also seems to be involved in calcium transport (37). Additionally, a member of the FLVCR family was described in *Leishmania major* (LmFLVCRb), capable of taking up porphyrins, heme inclusively, from the extracellular medium (38).

As previously described, a single FLVCR ortholog was identified in the genome of *Rhodnius prolixus*, a vector of Chagas disease (39). RpFLVCR has the 12 transmembrane canonical domains that are typical of these proteins. RpFLVCR knockdown impacts the survival of nymphs and adults, as well as oogenesis, embryogenesis and molting, during their life cycle, indicating that fluctuations in the levels of this protein can be deleterious to the insect development (39).

In this study, we present evidences that RpFLVCR acts as a heme transporter in *R. prolixus* and that heme efflux from the midgut lumen to the hemolymph is dependent on RpFLVCR. This process is critical for maintaining intestinal homeostasis and redox balance by modulating the expression of

genes involved in iron/heme metabolism and antioxidant protection. Furthermore, RpFLVCR knockdown also induces mitochondrial biogenesis with altered function, highlighting its paramount role in mitochondrial physiology in *R. prolixus*.

Results and Discussion

RpFLVCR acts as a heme exporter in Rhodnius prolixus

Heme in cells and extracellular fluids is rarely found as free heme, being more precisely described as “labile heme” which is exchangeable between distinct cellular targets including low molecular ligands or proteins, nucleic acids or phospholipid membranes (40). In vertebrates, two main mechanisms are known to be involved in the control of intracellular levels of labile heme: enzymatic degradation by heme oxygenase and heme transport provided by membrane transporters (41–43). Although heme degradation pathways have been documented in insects (17,18,44), the proteins responsible for the import and export of heme by the cells remains undescribed. Heme molecules provided in the bloodmeal reaches the hemolymph by crossing the midgut epithelium of the kissing bug *R. prolixus* (45), thus disclosing a mechanism that provides the transfer of heme molecules from the intestinal lumen of blood-sucking insects, through enterocytes to the hemolymph.

In the genome of *Rhodnius prolixus* a single FLVCR ortholog has been identified, which gene silencing strongly affected insect reproduction, development and survival after a blood meal (39). Thus, we decided to analyze whether, as in vertebrates, this transporter would be involved in the export of intracellular heme in *R. prolixus*.

Circulating levels of heme in hemolymph were measured in RpFLVCR-silenced females, being used as readout of heme export activity. Figure 1A shows that RpFLVCR-silenced females had a lower heme concentration in the hemolymph when compared to dsMAL-injected controls. If RpFLVCR works as a heme exporter, its knock-down would lead to an increase in intracellular heme, followed by heme degradation by heme oxygenase (HO). As observed in the midgut tissue, the levels of Rp-billiverdin, a breakdown product of heme catabolism (17), were higher in RpFLVCR-silenced females compared to control (Figure 1B). These results indicate that FLVCR silencing promotes increase of intracellular labile heme pool and supports the hypothesis that RpFLVCR acts

as a transmembrane exporter of heme from the intestinal epithelium to the hemolymph.

Knockdown of RpFLVCR modulates the expression of genes involved in heme and iron metabolism

It is well established in the literature that heme is a ubiquitous molecule involved in several cellular processes including signal transduction and transcriptional modulation (46–54). Thus, possible variations in heme levels, caused by RpFLVCR KD, could impact transcription of other genes involved with iron and heme metabolism. In this way, we seek to observe phenotypes that could be associated with variations in intracellular heme levels. We analyzed mRNA levels of genes encoding key proteins such as HO (heme degradation); Malvolium 1 and 2 (NRAMP orthologs from mammals involved in iron import), mitoferrin (Mfrn, a mitochondrial iron importer) and ferritin (Fer, an intracellular iron-storage protein). It was observed that RpFLVCR knockdown promoted an increase in the expression of HO, Mfrn, Fer and a decrease in iron importers MVL1 and MVL2 (Figure 2). These results suggest that the increase in HO expression may be a compensatory mechanism to control intracellular heme levels that were not exported due to RpFLVCR silencing. Increased expression of intracellular iron-binding proteins, such as ferritin (Fer) and mitoferrin (Mfrn) are required to prevent oxidative damage caused by iron from heme porphyrin ring breakage. On the other hand, with the increased availability of intracellular iron, import by MLV proteins became unnecessary, explaining the decrease in MLV expression (Figure 2B). Interestingly, HO KD caused an opposite effect in gene expression in comparison of RpFLVCR silencing, leading to increased expression of the RpFLVCR, MVL1 and MVL2 genes and decreased mRNA levels of ferritin (Figure 2C and 2D). These results suggested that reduction of HO expression increased intracellular heme but decrease “labile” iron levels. These results are consistent with increased RpFLVCR transcription working to remove the excess of heme. A decrease in free iron availability, would promote MVLs importers expression and conversely, lead to reduction in expression of iron chelating genes, such as ferritin and mitoferrin.

RpFLVCR depletion caused redox balance

Heme is known to be a pro-oxidant molecule due to its ability to propagate the formation of radical species (ROS). It can promptly react to organic hydroperoxides producing alkoxyl or peroxyl lipid radicals, thus increasing lipid peroxidation (6). Indeed, midguts of RpFLVCR-silenced insects showed increased fluorescence of the redox-sensitive probe DHE (Figure 3A), suggesting redox imbalance. This conclusion was further supported by TBARS assay analysis for lipid peroxidation levels in the midgut, showing increase in lipid peroxidation in dsRpFLVCR - injected females (Figure 3C). The impact on the midgut redox balance was also monitored by quantification of mRNA levels of antioxidant genes, usually induced in response to high levels of ROS, such as catalase (CAT), glutathione peroxidase (PHGPx), thioredoxin reductase (TrxR) and peroxiredoxins (Prx). Corroborating DHE-fluorescence and TBARS data, females silenced for RpFLVCR gene showed an increase in the antioxidant enzymes mRNA levels when compared to control insects (Figure 3D). Surprisingly, HO KD had no effect on the cellular redox status (Figure 3B, C and D). This result could be explained by either one of the mechanisms: i) Rp FLVCR mediated export of the remained labile heme left undegraded upon HO KD; ii) by the oxidative insult to the intestinal cells upon RpFLVCR KD being more derived from iron produced by heme cleavage by HO than by increased labile heme itself. In fact, it has already been shown that ferritin knockdown, the major intracellular iron chelator, caused a dramatic increase of oxidant production in *R. prolixus* midgut (39). Choosing between these alternative hypotheses is a central point for future research on redox metabolism in blood-sucking insects.

RpFLVCR silencing induces mitochondrial biogenesis with altered function

Hemeproteins represent a significant fraction of the electron transport system (ETS) components, implying mitochondria as the main site of iron and heme storage in eukaryotic cells (55). Therefore, change in heme levels in animals silenced for RpFLVCR can interfere with energy metabolism and mitochondrial function. To test this hypothesis, we first determined mitochondrial content in the intestinal cells of the silenced animals through MitoTracker Green fluorescence microscopy, as this probe accumulates

preferably in mitochondria. We observed that 5 days after blood feeding the females silenced for the RpFLVCR gene showed a higher fluorescence than the control insects or insects silenced for HO, suggesting an increase in the mitochondrial content (Figure 4A and 4B). This result was corroborated by the increased activity of citrate synthase, an important tricarboxylic acid cycle enzyme classically used as an index of mitochondrial content (56), in the midguts of RpFLVCR-silenced insects (Figure 4C). Surprisingly, cytochrome c oxidase activity was strongly reduced specifically in RpFLVCR silenced insects, suggesting that *de novo* mitochondrial biogenesis generates organelles with lower electron flux and respiratory rates (Figure 4D). To gain further insights on the mechanisms behind FLVCR KD-induced mitochondrial expansion, we measured expression of genes that have a central role in mitochondrial biogenesis. Mitochondrial transcription factor A (TFAM), is a nuclear-encoded protein responsible for the transcription and translation of mitochondrial DNA (mtDNA) (57,58) and Delg (CG6338; *Drosophila* Ets like gene) is a close homologue to mammalian NRF-2α that is critical to adjust mitochondrial abundance (59), being their expression frequently associated to mitochondria biogenesis. The quantification of *R. prolixus* orthologs of these genes revealed that RpFLVCR KD promotes an increase in mRNA levels of both genes, whereas HO KD did not (Figure 4 E and F), suggesting that RpFLVCR silencing increase mitochondrial mass in the midgut in association increased with TFAM and DELG expression.

As RpFLVCR KD promotes mitochondrial biogenesis, and mitochondria are a source of ROS in eukaryotic cells (60–62), we noticed that the RpFLVCR KD-induced increase in DHE fluorescence upon RpFLVCR KD was strongly reduced by MitoTempo (Figure 5A). Conceivably, increased cellular oxidant production upon RpFLVCR silencing results from reduced cytochrome c oxidase activity, then facilitating mitochondrial electron leak and superoxide production. In this sense, mitochondria represent the major site of cellular oxidant production (63) and altered mitochondrial electron flux promoted by RpFLVCR silencing (Figures 4 and 5) might contribute to superoxide production. Altogether, these results indicate that the redox imbalance observed in RpFLVCR-silenced insects is derived from mitochondrial oxidant production.

However, the mechanism by which RpFLVCR KD modulates mitochondrial biogenesis and the relationship of this phenomenon with the increase in cellular oxidant levels still needs to be clarified.

Recently, it was shown in two elegant reports that the mammalian FLVCR works as a choline transporter instead of heme transporter (64,65). Evidence for excluding a role for this membrane protein on heme transport came from experiments that cells KO for FLVCR do not impact HO expression, as well as other heme and iron-related genes (64). The same report also observes alterations in mitochondria comparable to those found here, but instead, attributed this phenotype to deficient phospholipid biosynthesis. Here, we have not addressed a role of RpFLVCR in choline transport, an interesting possibility that could well explain part of the deleterious effects of RpFLVCR on insect oogenesis, embryo and nymph development (39), all biological phenomena that need a large amount of phosphatidylcholine. However, the results presented here reveal a robust impact of FLVCR on heme and iron cell biology that is unlikely to be explained by choline deficiency alone. Therefore, it is possible that FLVCR is a moonlight transporter, with the choline transport being the function for most cell types that live in the presence of heme concentrations in the low micromolar range (used in the experiments of Kenny et al (64), while cells that physiologically face higher heme concentrations may use FLVCR as a low affinity but functional transporter. Vertebrate blood has about 150 mg/ml hemoglobin, which, upon complete digestion would result in ~5 mM heme. This bold number suggests that RpFLVCR may well be such a moonlight heme transporter. As hematophagy has arisen independently multiple times during the evolution of insects (66), further investigation with other hematophagous insect species is needed to see how frequent is the recruitment of FLVCR homologs as a key player in the adaptation of the insect to hematophagy, as seem to be the case for *Rhodnius prolixus* intestinal epithelia.

Experimental procedures

Experimental Insects

Insects were taken from a colony of *R. prolixus* maintained at 28°C and 70–90% relative humidity under a photoperiod of 12 h of light/12 h of dark. All animals used in this work were mated females fed on rabbit blood at 3-week intervals. *R. prolixus* females injected with dsRNA were kept in individual vials maintained at the same conditions.

Ethics Statement

All animal care and experimental protocols were conducted following the guidelines of the institutional care and use committee (Committee for Evaluation of Animal Use for Research from the Federal University of Rio de Janeiro, CAUAP-UFRJ), which are based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals (ISBN 0-309-05377-3). The protocols were approved by the Committee for Evaluation of Animal Use for Research from the Federal University of Rio de Janeiro under registry number IBQM0123/22. Dedicated technicians in the animal facility at the Instituto de Bioquímica Médica Leopoldo de Meis (UFRJ) carried out all protocols related to rabbit husbandry under strict guidelines to ensure careful and consistent animal handling.

Tissue Isolation and RNA Extraction

Posterior midguts from blood-fed females were dissected at different days after blood meal. Total RNA was extracted from individual tissues or pools of 3–5 midguts using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. Complementary DNA was synthesized using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, United States).

dsRNA Synthesis and Gene Silencing Assays

Fragments of 320–400 bp were amplified by PCR using cDNA from midgut epithelia from blood-fed females (48 h after feeding) produced as described above. The following conditions were used for amplification: one cycle for 5 min at 95°C, followed by 40 cycles of 30 s a 95°C, 30 s at 63°C, and 1 min at 72°C, with a final step of 10 min at 72°C. The oligonucleotides used for amplification of templates for dsRNA synthesis are listed at Supporting Table S1. Amplified cDNAs were used as a template for dsRNA synthesis using a MEGAScript RNAi kit (Ambion Inc., Austin, TX, United States) according to the manufacturer's instructions. The maltose-binding protein (MAL) gene from *Escherichia coli* (gene identifier 7129408) in a pBlueScript KS (Stratagene) was amplified by PCR using T7 minimal promoter primers under the following conditions: one cycle for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 15 s at 45°C, and 45 s at 72°C, with a final step of 10 min at 72°C. The PCR product produced was used as a template for MAL dsRNA synthesis used as a control in the gene silencing assays. Following *in vitro* synthesis, all dsRNAs were purified according to the manufacturer's instructions. For gene silencing, 1 µg of dsRNA specific for RpFLVCR (dsRpFLVCR), Heme Oxygenase (dsHO) or MAL (dsMAL) were injected into starved insect hemocoele. Insects were fed on rabbit blood 48 h after injection. At different times after feeding, midguts were dissected, total RNA was extracted as described above, and gene silencing efficiency was evaluated by quantitative RT-PCR. The efficiency of RpFLVCR and RpHO silencing is shown in Supporting figure S1.

Quantitative RT-PCR Analysis

Quantitative gene amplification (qPCR) was performed with the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, United States) using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, United States) under the following conditions: one cycle for 10 min at 95°C, followed by forty cycles of 15 s at 95°C and 45 s at 60°C. PCR amplification was performed using the oligonucleotides specified in Supporting Table S2. *R. prolixus* elongation factor 1 gene (RPRC007684) expression was used as an internal control for normalization (67). The relative expression values based on $2^{-\Delta\Delta C_T}$were used in silenced genes analyses (68). Differences

were considered significant when $p < 0.05$. The relative expression values based on $2^{-\Delta\Delta C_T}$ were used only for graphic construction.

Quantification of Heme Content in the hemolymph

Hemolymph was collected 3 days after blood feeding from previously silenced females in tubes containing a few crystals of phenylthiourea, by cutting a leg, and applying a gentle pressure to the insect abdomen. Qualitative analyses of heme levels were assessed by diluting a hemolymph aliquot of 10 µL in 490 µL PBS pH 7.4, and the light absorption spectra was analyzed between 300 nm and 800 nm in a Shimadzu UV-2550 spectrophotometer. Absolute quantification of total heme was determined by the alkaline pyridine-hemochrome method, using the reduced minus oxidized spectra as described elsewhere (69).

Quantification of biliverdin in the midgut

Two days prior to the blood meal, adult females were injected with 1 µL of dsRNA (1 µg/µL) into the thoracic cavity. Five individual midguts were dissected 3 days after feeding, and individually homogenized with 200 µL 5% acetonitrile and 0.05% TFA, pH 2.0, (used as solvent for the HPLC column), centrifuged for 15 min at 12,000×g and the supernatant was applied onto a Shimadzu CLC-ODS C18 column (15 mm × 22 cm) equilibrated with the same solvent, using a flow rate of 0.4 mL/min using a LC-10AT device (Shimadzu, Tokyo, Japan) equipped with a diode array detector (SPDM10A). After 10 min, an acetonitrile linear gradient (5–80%) was applied for 10 min, followed by 20 min of 80% acetonitrile with 0.05% TFA, pH 2.0. Biliverdin peak was identified and quantified as previously described (17).

Analysis of oxidant status in different tissues

Lipid peroxidation levels in hemolymph of silenced females were assessed 3 days after blood meal by quantifying the levels of thiobarbituric acid reactive substances assay (TBARS) at 532 nm in a Molecular Devices Spectra Max M5 spectrophotometer as described elsewhere (70). Also, to evaluate oxidant levels in the midgut, starved females were injected with 1 µg of RpFLVCR dsRNA, HO dsRNA or MAL dsRNA (control). Two days after injection insects were fed with rabbit blood and their wings, legs and dorsal cuticle were removed by dissection

and the insect hemocoel was filled with 50 μM of the oxidant-sensitive fluorophore dihydroethidium (hydroethidine; DHE; Invitrogen, Carlsbad, CA, United States) diluted in Leibovitz-15 media containing 5% fetal bovine serum or with DHE and 5μM MitoTEMPO (Santa Cruz, Dallas, TX, United States). The midguts were incubated in the dark at 28°C for 20 min, washed with 0.15 M NaCl, and immediately transferred to a glass slide for fluorescence microscopy analysis, as previously described (71). Quantitative evaluation of fluorescence levels was performed by acquiring images under identical conditions using an objective of 20X and 90 ms exposure time in each experiment. The images were acquired in a Zeiss Observer.Z1 with a Zeiss Axio Cam MrM Zeiss and the data were analyzed using AxioVision version 4.8 software. The filter set (excitation BP 546/12 nm; beam splitter FT 580 nm; emission LP 590 nm) was used for DHE labeling.

Analysis of Mitochondrial content

Mitochondrial content was determined by measuring the activity of citrate synthase (CS) and by MitoTracker Green fluorescence. CS activity was assayed according to the method described by Hansen and Sidell (1983)(72), with modifications. Pools of two midguts were homogenized in 200 μL of saline solution. After 2 min of decantation, 40 μL of supernatant was incubated with 7.5 mM Tris buffer (pH 8.0) containing 50 μM DTNB (5,5′-dithiobis (2-nitrobenzoic acid)) (Sigma-Aldrich, MI, United States), 300 μM acetyl-CoA and 1 mM oxaloacetate. Immediately, DTNB reduction was measured for 10 min at 412 nm. The specific activity was calculated using the reduced DTNB molar extinction coefficient (ϵ =13.6 $mM^{-1}\cdot cm^{-1}$). MitoTracker Green is a mitochondrial selective probe that binds covalently to mitochondrial proteins by reacting with cysteine residues and accumulates in the mitochondrial matrix (73–75). This reaction is independent of mitochondrial membrane potential and is widely used in literature to represent mitochondrial mass (56,76). Starved adult females were injected with 1 μL of dsRpFLVCR, dsHO or dsMAL into the thoracic cavity. Two days after injection, the insects were fed on blood. Mitochondria were stained with 100 μM MitoTracker Green FM (Invitrogen, Carlsbad, CA, United States) diluted in Lebovitz-15 media containing 5% fetal bovine serum for 30 minutes and with DAPI (0,1mg/mL) for 10 minutes at 28°C in the dark, 5 days

after feeding. Afterwards, the midguts were washed with 0.15 M NaCl, and immediately transferred to a glass slide for fluorescence microscopy analysis. The images were acquired in an Olympus IX81 microscope and a CellR MT20E Imaging Station equipped with an IX2-UCB controller and an ORCAR2 C10600 CCD camera (Hammamatsu). Image processing was performed with the Xcellence RT version 1.2 Software.

Cytochrome c Oxidase Activity

Activity of cytochrome *c* oxidase was measured in a total reaction volume of 1 mL, using a Shimadzu spectrophotometer UV-visible 2450 (Shimadzu Scientific Instruments, Tokyo, Japan). Enzyme activity was measured by following the decrease in absorbance caused by the oxidation of ferrocytochrome c (ϵ = 18.5 mM^{-1} cm^{-1}) (77) modified according to Gaviraghi (78). The reaction mixture consisted of 100 mM potassium phosphate (pH 7.4), 50 mM reduced cytochrome c. The reaction was initiated by the addition of freeze-thawed mitochondria (80 µg of protein), and the reduction in absorbance at 550 nm was monitored. KCN (1 mM) was added to inhibit cytochrome *c* oxidase activity, which was considered as the cyanide-sensitive rate of cytochrome *c* oxidation. The data are expressed as nmol of reduced cytochrome *c*/min/mg protein of isolated mitochondria.

Statistical analysis

All analyses were performed with the GraphPad Prism statistical software package (Prism version 6.0, GraphPad Software,Inc., La Jolla, CA). Significant differences are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.), and the type of test used in each analysis is described in its respective figure legend.

Supporting information - This article contains supporting information.

Author contributions - Author contributions – ABWN, MFO and GOPS conceived and designed the experiments; ABWN, MTA and MPO performed the

experiments; ABW-N, MFO, PLO and GOPS data analysis; .ABWN writing – original draft; All authors review & editing; GOPS supervision.

Acknowledgments - We thank all members of the Laboratório de Bioquímica de Artrópodos Hematófagos for critical suggestions and S. R. Cássia for providing technical assistance.

Conflict of interest - The authors declare that they have no conflicts of interest with the contents of this article.

Funding - This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (MFO, PLO, GOPS), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (MFO, PLO, GOPS), and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) (ABWN, MFO, PLO, GOPS).

References

1. PONKA P. Cell Biology of Heme. Am J Med Sci. 1999 Oct;318(4):241.
2. Kim HJ, Khalimonchuk O, Smith PM, Winge DR. Structure, function, and assembly of heme centers in mitochondrial respiratory complexes. Vol. 1823, Biochimica et Biophysica Acta - Molecular Cell Research. 2012. p. 1604–16.
3. Shimizu T, Huang D, Yan F, Stranava M, Bartosova M, Fojtíková V, et al. Gaseous O2, NO, and CO in Signal Transduction: Structure and Function Relationships of Heme-Based Gas Sensors and Heme-Redox Sensors. Vol. 115, Chemical Reviews. American Chemical Society; 2015. p. 6491–533.
4. TAPPEL AL. Unsaturated lipide oxidation catalyzed by hematin compounds. J Biol Chem. 1955 Dec 1;217(2):721–33.
5. Gutteridge JMC, Smitht A. Antioxidant protection by haemopexin of haem-stimulated lipid peroxidation. Vol. 256, Biochem. J. 1988.
6. Ryter SW, Tyrrell RM. Hypothesis Paper THE HEME SYNTHESIS AND DEGRADATION PATHWAYS: ROLE IN OXIDANT SENSITIVITY Heme Oxygenase has Both Pro-and Antioxidant Properties. 2000.
7. Schmitt TH, Frezzatti WA, Schreier S. Hemin-Induced Lipid Membrane Disorder and Increased Permeability: A Molecular Model for the Mechanism of Cell Lysis. Arch Biochem Biophys. 1993 Nov;307(1):96–103.
8. Rajagopal A, Rao AU, Amigo J, Tian M, Upadhyay SK, Hall C, et al. Haem homeostasis is regulated by the conserved and concerted functions of HRG-1 proteins. Nature. 2008 Jun 19;453(7198):1127–31.
9. Chen C, Samuel TK, Krause M, Dailey HA, Hamza I. Heme utilization in the Caenorhabditis elegans hypodermal cells is facilitated by heme-responsive gene-2. Journal of Biological Chemistry. 2012 Mar 16;287(12):9601–12.

10. White C, Yuan X, Schmidt PJ, Bresciani E, Samuel TK, Campagna D, et al. HRG1 is essential for heme transport from the phagolysosome of macrophages during erythrophagocytosis. Cell Metab. 2013;17(2).

11. Chiabrando D, Vinchi F, Fiorito V, Mercurio S, Tolosano E. Heme in pathophysiology: A matter of scavenging, metabolism and trafficking across cell membranes. Front Pharmacol. 2014;5:1–24.

12. Donegan RK, Moore CM, Hanna DA, Reddi AR. Handling heme: The mechanisms underlying the movement of heme within and between cells. Vol. 133, Free Radical Biology and Medicine. Elsevier Inc.; 2019. p. 88–100.

13. Graça-Souza A V., Maya-Monteiro C, Paiva-Silva GO, Braz GRC, Paes MC, Sorgine MHF, et al. Adaptations against heme toxicity in blood-feeding arthropods. Vol. 36, Insect Biochemistry and Molecular Biology. Elsevier Ltd; 2006. p. 322–35.

14. Oliveira MF, Kycia SW, Gomez A, Kosar AJ, Bohle DS, Hempelmann E, et al. Structural and morphological characterization of hemozoin produced by Schistosoma mansoni and Rhodnius prolixus. FEBS Lett. 2005 Nov 7;579(27):6010–6.

15. Stiebler R, Timm BL, Oliveira PL, Hearne GR, Egan TJ, Oliveira MF. On the physico-chemical and physiological requirements of hemozoin formation promoted by perimicrovillar membranes in Rhodnius prolixus midgut. Insect Biochem Mol Biol. 2010 Mar;40(3):284–92.

16. Davenport M, Alvarenga PH, Shao L, Fujioka H, Bianconi ML, Oliveira PL, et al. Identification of the Aedes aegypti peritrophic matrix protein AeIMUCI as a heme-binding protein. Biochemistry. 2006 Aug 8;45(31):9540–9.

17. Paiva-Silva GO, Cruz-Oliveira C, Nakayasu ES, Maya-Monteiro CM, Dunkov BC, Masuda H, et al. A heme-degradation pathway in a blood-sucking insect [Internet]. 2006. Available from: www.pnas.orgcgidoi10.1073pnas.0602224103

18. Pereira LOR, Oliveira PL, Almeida IC, Paiva-Silva GO. Biglutaminyl-biliverdin IX alpha as a heme degradation product in the dengue fever insect-vector Aedes aegypti. Biochemistry. 2007 Jun 12;46(23):6822–9.

19. Braz GRC, Coelho HSL, Masuda H, Oliveira PL. A missing metabolic pathway in the cattle tick Boophilus microplus. 1999; Available from: http://biomednet.com/elecref/0960982200900703

20. Braz GRC, Moreira MF, Masuda H, Oliveira PL. Rhodnius heme-binding protein (RHBP) is a heme source for embryonic development in the blood-sucking bug Rhodnius prolixus (hemiptera, reduviidae). Insect Biochem Mol Biol. 2002;32(4).

21. Lara FA, Pohl PC, Gandara AC, Ferreira JDS, Nascimento-Silva MC, Bechara GH, et al. ATP binding cassette transporter mediates both heme and pesticide detoxification in tick midgut cells. PLoS One. 2015 Aug 10;10(8).

22. Eggleston H, Adelman ZN. Transcriptomic analyses of Aedes aegypti cultured cells and ex vivo midguts in response to an excess or deficiency of heme: A quest for transcriptionally-regulated heme transporters. BMC Genomics. 2020 Aug 31;21(1).

23. Tailor CS, Willett BJ, Kabat D. A Putative Cell Surface Receptor for Anemia-Inducing Feline Leukemia Virus Subgroup C Is a Member of a Transporter Superfamily. Vol. 73, JOURNAL OF VIROLOGY. 1999.

24. Quigley JG, Burns CC, Anderson MM, Lynch ED, Sabo KM, Overbaugh J, et al. Cloning of the cellular receptor for feline leukemia virus subgroup C (FeLV-C), a retrovirus that induces red cell aplasia. Blood. 2000;95(3).

25. Quigley JG, Yang Z, Worthington MT, Phillips JD, Sabo KM, Sabath DE, et al. Identification of a Human Heme Exporter that Is Essential for Erythropoiesis through a balance among heme biosynthesis, utilization for hemoproteins, and catabolism by the microsomal enzyme, heme oxygenase (HO) (Ponka, 1997), to gener-ate carbon monoxide, iron, and biliverdin [Internet]. Vol. 118, Cell. 2004. Available from: http://source.stanford.edu

26. Pao SS, Paulsen IT, Saier MH. Major Facilitator Superfamily. Vol. 62, MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS. 1998.

27. Chiabrando D, Marro S, Mercurio S, Giorgi C, Petrillo S, Vinchi F, et al. The mitochondrial heme exporter FLVCR1b mediates erythroid differentiation. J Clin Invest [Internet]. 2012;122:4569. Available from: http://www.jci.org

28. Keel SB, Doty RT, Yang Z, Quigley JG, Chen J, Knoblaugh S, et al. A heme export protein is required for red blood cell differentiation and iron homeostasis. Science (1979). 2008;319(5864).

29. Khan AA, Quigley JG. Control of intracellular heme levels: Heme transporters and heme oxygenases. Vol. 1813, Biochimica et Biophysica Acta - Molecular Cell Research. 2011.

30. Khan AA, Quigley JG. Heme and FLVCR-related transporter families SLC48 and SLC49. Vol. 34, Molecular Aspects of Medicine. 2013.

31. Vinchi F, Ingoglia G, Chiabrando D, Mercurio S, Turco E, Silengo L, et al. Heme exporter FLVCR1a regulates heme synthesis and degradation and controls activity of cytochromes P450. Gastroenterology. 2014;146(5).

32. Fiorito V, Forni M, Silengo L, Altruda F, Tolosano E. Crucial role of FLVCR1a in the maintenance of intestinal heme homeostasis. Antioxid Redox Signal. 2015 Dec 20;23(18):1410–23.

33. Petrillo S, Chiabrando D, Genova T, Fiorito V, Ingoglia G, Vinchi F, et al. Heme accumulation in endothelial cells impairs angiogenesis by triggering paraptosis. Cell Death Differ. 2018 Mar 1;25(3):573–88.

34. Mercurio S, Petrillo S, Chiabrando D, Bassi ZI, Gays D, Camporeale A, et al. The heme exporter Flvcr1 regulates expansion and differentiation of committed erythroid progenitors by controlling intracellular heme accumulation. Haematologica. 2015;100(6):720–9.

35. Ponka P, Sheftel AD, English AM, Scott Bohle D, Garcia-Santos D. Do Mammalian Cells Really Need to Export and Import Heme? Vol. 42, Trends in Biochemical Sciences. Elsevier Ltd; 2017. p. 395–406.

36. Duffy SP, Shing J, Saraon P, Berger LC, Eiden M V., Wilde A, et al. The Fowler Syndrome-Associated Protein FLVCR2 Is an Importer of Heme. Mol Cell Biol. 2010 Nov 1;30(22):5318–24.

37. Brasier G, Tikellis C, Xuereb L, Craigie J, Casley D, Kovacs CS, et al. Novel hexad repeats conserved in a putative transporter with restricted expression in cell types associated with growth, calcium exchange and homeostasis. Exp Cell Res. 2004 Feb 1;293(1):31–42.

38. Cabello-Donayre M, Orrego LM, Herráez E, Vargas P, Martínez-García M, Campos-Salinas J, et al. Leishmania heme uptake involves LmFLVCRb, a novel porphyrin transporter essential for the parasite. Cellular and Molecular Life Sciences. 2020;77(9).

39. Walter-Nuno AB, Taracena ML, Mesquita RD, Oliveira PL, Paiva-Silva GO. Silencing of iron and heme-related genes revealed a paramount role of iron in the physiology of the hematophagous vector Rhodnius prolixus. Front Genet. 2018;9(FEB).

40. Gouveia Z, Carlos AR, Yuan X, Aires-da-Silva F, Stocker R, Maghzal GJ, et al. Characterization of plasma labile heme in hemolytic conditions. FEBS Journal. 2017 Oct 1;284(19):3278–301.

41. Hamza I, Dailey HA. One ring to rule them all: Trafficking of heme and heme synthesis intermediates in the metazoans. Vol. 1823, Biochimica et Biophysica Acta - Molecular Cell Research. 2012.

42. Chambers IG, Willoughby MM, Hamza I, Reddi AR. One ring to bring them all and in the darkness bind them: The trafficking of heme without deliverers. Vol. 1868, Biochimica et Biophysica Acta - Molecular Cell Research. 2021.

43. Korolnek T, Hamza I. Like iron in the blood of the people: The requirement for heme trafficking in iron metabolism. Vol. 5 JUN, Frontiers in Pharmacology. 2014.

44. Caiaffa CD, Stiebler R, Oliveira MF, Lara FA, Paiva-Silva GO, Oliveira PL. Sn-protoporphyrin inhibits both heme degradation and hemozoin

formation in Rhodnius prolixus midgut. Insect Biochem Mol Biol. 2010 Dec;40(12):855–60.

45. Dansa-Petretski M, Ribeiro JMC, Atella GC, Masuda H, Oliveira PL. Antioxidant role of Rhodnius prolixus heme-binding protein. Protection against heme-induced lipid peroxidation. Journal of Biological Chemistry. 1995;270(18).

46. Zhang L. Heme Biology: The secret life of heme in regulating diverse biological processes. First Edition. Singapure: World Scientific; 2011. 139–196 p.

47. Shimizu T, Lengalova A, Martínek V, Martínková M. Heme: Emergent roles of heme in signal transduction, functional regulation and as catalytic centres. Chem Soc Rev. 2019 Dec 21;48(24):5624–57.

48. Bottino-Rojas V, Talyuli OAC, Jupatanakul N, Sim S, Dimopoulos G, Venancio TM, et al. Heme signaling impacts global gene expression, immunity and dengue virus infectivity in Aedes aegypti. PLoS One. 2015 Aug 14;10(8).

49. Woldt E, Sebti Y, Solt LA, Duhem C, Lancel S, Eeckhoute J, et al. Rev-erb-α modulates skeletal muscle oxidative capacity by regulating mitochondrial biogenesis and autophagy. Nat Med. 2013 Aug;19(8):1039–46.

50. Mense SM, Zhang L. Heme: a versatile signaling molecule controlling the activities of diverse regulators ranging from transcription factors to MAP kinases. Cell Res. 2006 Aug 8;16(8):681–92.

51. Suzuki H, Tashiro S, Hira S, Sun J, Yamazaki C, Zenke Y, et al. Heme regulates gene expression by triggering Crm1-dependent nuclear export of Bach1. EMBO Journal. 2004 Jul 7;23(13):2544–53.

52. Ogawa. Heme mediates deprepression of Maf recognition element through direct binding to transcription repressor Bach1. 2001;

53. Zhang L, Guarentel L. Heme binds to a short sequence that serves a regulatory function in diverse proteins. Vol. 14, The EMBO Journal. 1995.

54. Zhang L. Heme Biology: Heme acts as a versatile signalling molecule regulating diverse biological processses. Second Edition. Zhang L, editor. Dallas : World Scientific ; 2020.

55. Jhurry ND, Chakrabarti M, McCormick SP, Holmes-Hampton GP, Lindahl PA. Biophysical investigation of the ironome of human Jurkat cells and mitochondria. Biochemistry. 2012 Jul 3;51(26):5276–84.

56. Larsen S, Nielsen J, Hansen CN, Nielsen LB, Wibrand F, Stride N, et al. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. Journal of Physiology. 2012 Jul;590(14):3349–60.

57. Picca A, Lezza AMS. Regulation of mitochondrial biogenesis through TFAM-mitochondrial DNA interactions. Useful insights from aging and calorie restriction studies. Vol. 25, Mitochondrion. Elsevier; 2015. p. 67–75.

58. Cardanho-Ramos C, Morais VA. Mitochondrial biogenesis in neurons: How and where. Vol. 22, International Journal of Molecular Sciences. MDPI; 2021.

59. Baltzer C, Tiefenböck SK, Marti M, Frei C. Nutrition controls mitochondrial biogenesis in the Drosophila adipose tissue through Delg and cyclin D/Cdk4. PLoS One. 2009 Sep 9;4(9).

60. Nickel A, Kohlhaas M, Maack C. Mitochondrial reactive oxygen species production and elimination. Vol. 73, Journal of Molecular and Cellular Cardiology. Academic Press; 2014. p. 26–33.

61. Starkov AA. The role of mitochondria in reactive oxygen species metabolism and signaling. In: Annals of the New York Academy of Sciences. Blackwell Publishing Inc.; 2008. p. 37–52.

62. Murphy MP. How mitochondria produce reactive oxygen species. Vol. 417, Biochemical Journal. 2009. p. 1–13.

63. Boveris A, Oshino N, Chance B. The Cellular Production of Hydrogen Peroxide. Vol. 128, Biochem. J. 1972.

64. Kenny TC, Khan A, Son Y, Yue L, Heissel S, Sharma A, et al. Integrative genetic analysis identifies FLVCR1 as a plasma-membrane choline transporter in mammals. Cell Metab. 2023 Jun 6;35(6):1057-1071.e12.

65. Tsuchiya M, Tachibana N, Nagao K, Tamura T, Hamachi I. Organelle-selective click labeling coupled with flow cytometry allows pooled CRISPR screening of genes involved in phosphatidylcholine metabolism. Cell Metab. 2023 Jun;35(6):1072-1083.e9.

66. Marquardt WC, Kondratieff BC. Biology of Disease Vectors. Second Edition. Burlington: Elsevier Academic Press; 2005.

67. Majerowicz D, Alves-Bezerra M, Logullo R, Fonseca-De-Souza AL, Meyer-Fernandes JR, Braz GRC, et al. Looking for reference genes for real-time quantitative PCR experiments in Rhodnius prolixus (Hemiptera: Reduviidae). Insect Mol Biol. 2011 Dec;20(6):713–22.

68. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 2001;25(4):402–8.

69. Falk JE. Porphyrin and metalloporphyrins: their general, physical and coordination chemistry, and laboratory methods. Elsevier Publishing Company; 1964.

70. Liedtke AJ, Mahar CQ, Ytrehus K, Mjcs OD. Estimates of free-radical production in rat and swine hearts: method and application of measuring malondialdehyde levels in fresh and frozen myocardium. Vol. 179, Basic Res. 1984.

71. Walter-Nuno AB, Oliveira MP, Oliveira MF, Gonçalves RL, Ramos IB, Koerich LB, et al. Silencing of maternal heme-binding protein causes embryonic mitochondrial dysfunction and impairs embryogenesis in the blood sucking insect rhodnius prolixus. Journal of Biological Chemistry. 2013;288(41).

72. Hansen CA, Sidell BD. Atlantic hagfish cardiac muscle: metabolic basis of tolerance to anoxia [Internet]. 1983. Available from: www.physiology.org/journal/ajpregu

73. Presley AD, Fuller KM, Arriaga EA. MitoTracker Green labeling of mitochondrial proteins and their subsequent analysis by capillary electrophoresis with laser-induced fluorescence detection. Journal of Chromatography B. 2003 Aug;793(1):141–50.

74. Poot M, Zhang YZ, Krämer JA, Wells KS, Jones LJ, Hanzel DK, et al. Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. Journal of Histochemistry & Cytochemistry. 1996 Dec 25;44(12):1363–72.

75. Soultanakis RP, Melamede RJ, Bespalov IA, Wallace SS, Beckman KB, Ames BN, et al. Fluorescence detection of 8-oxoguanine in nuclear and mitochondrial DNA of cultured cells using a recombinant Fab and confocal scanning laser microscopy. Free Radic Biol Med. 2000 Mar;28(6):987–98.

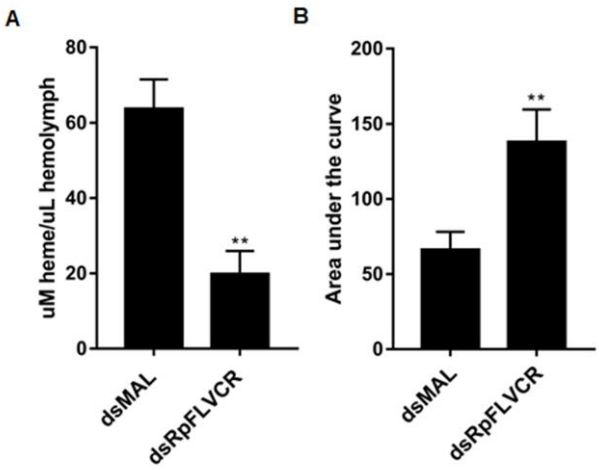
76. Agnello M, Morici G, Rinaldi AM. A method for measuring mitochondrial mass and activity. Cytotechnology. 2008 Mar;56(3):145–9.

77. Kirby DM, Thorburn DR, Turnbull DM, Taylor RW. Biochemical Assays of Respiratory Chain Complex Activity. Vol. 80, Methods in Cell Biology. 2007. p. 93–119.

78. Gaviraghi A, Correa Soares JBR, Mignaco JA, Fontes CFL, Oliveira MF. Mitochondrial glycerol phosphate oxidation is modulated by adenylates through allosteric regulation of cytochrome c oxidase activity in mosquito flight muscle. Insect Biochem Mol Biol. 2019 Nov 1;114.

1

2 ***Figure and Legends***



3

4 **Figure 1: FLVCR acts as a heme exporter in *R.prolixus***. Adult females were
5 injected with dsRpFLVCR or dsMAL (control group) and fed on blood as
6 described. (A) Quantification of heme present in the hemolymph of females
7 injected with dsRpFLVCR and dsMAL. The amount of heme present in
8 hemolymph was determined by the alkaline pyridine method. Data represent
9 mean ± S.E. for 10 individual determinations per experiment of four
10 independent experiments. Statistical analysis between the two groups was
11 performed using Student's *t* test. (B) Quantification of biliverdin present in the
12 midgut of silenced RpFLVCR insects. The amount of biliverdin were measured
13 as described in section 2, by HPLC. Data represent mean ± S.E. for n=5 of
14 three independent experiments. Statistical analysis between the two groups
15 was performed using Student's *t* test. ** $p < 0.01$

16

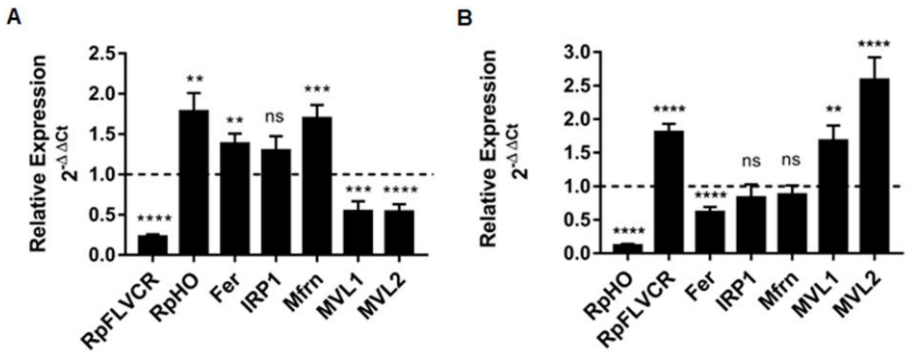


Figure 2: FLVCR silencing is able to modulate genes from heme and iron metabolism. Starved adult females were injected with dsRpRFLVCR (A) or dsRpHO (B). Insects were fed on blood 48h after injection. Total RNA was extracted from foregut and hindgut and mRNA levels of genes involved in iron and heme metabolism analyzed by quantitive RT-PCR. The EF-1 gene was used as an endogenous control. The result was normalized to animals injected with dsMAL (dashed line). Data represent mean ± S.E. (n= 14-15) of three independent experiments. Statistical analyzes were performed using the Student's t test (experimental versus their respective controls, dsMal). *p<0.05; ** p<0.01; *** p<0.001; ****p<0.0001. RpFLVCR, Feline Leukemia Virus C receptor FLVCR; RpHO, Heme oxygenase; Fer, Ferritin; IRP1, Iron Regulatory Protein 1; Mfrn, mitoferrin; MVL1, malvolio 1; MVL2, malvolio 2

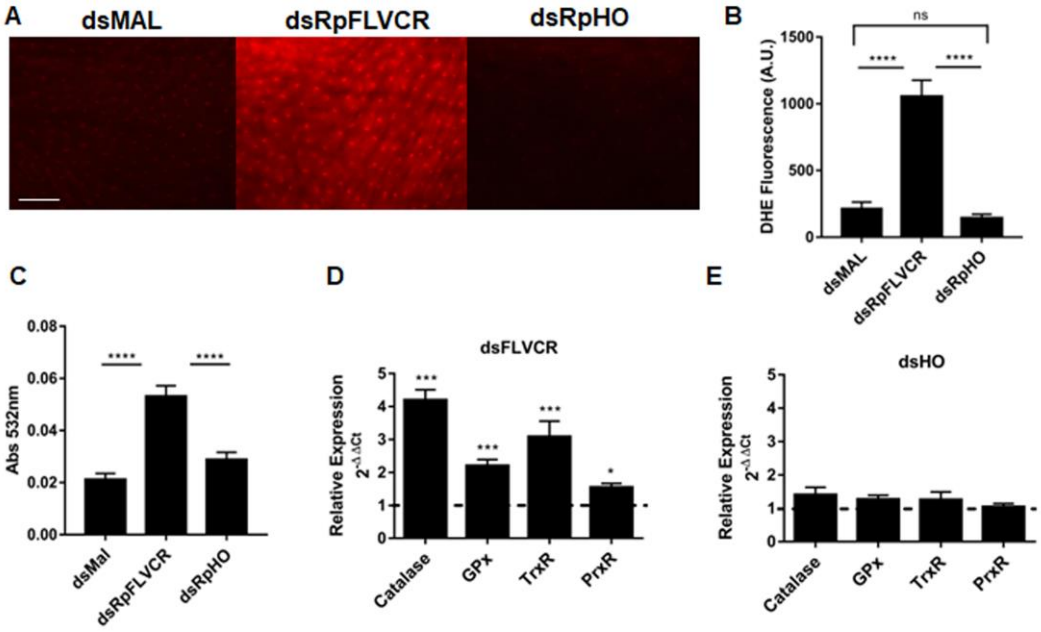


Figure 3. FLVCR silencing increase ROS levels in the midgut cells. (A) ROS levels were evaluated from oxidized DHE fluorescence in cells from blood-fed females previously injected with dsRpFLVCR, dsRpHO or dsMal (control). Representative images of insects for each experimental condition are shown, scale bar 2 µm. Quantitative analysis of DHE fluorescence of 10 individual intestines in three independent experiments is shown in the graph. Data shown are mean ± S.E., **** $p<0.0001$ for One-way ANOVA with Tukey's post test. (B) Lipid peroxidation of hemolymph was quantified by the TBARS assay as described. Data shown are mean ± S.E. (n= 4), at three independent experiments. **** $p<0.0001$ for One-way ANOVA with Tukey's post test. (C-D). Expression of selected antioxidant genes was evaluated by quantitative RT-PCR in insects silenced either for RpFLVCR (C) or RpHO (D). The result was normalized to animals injected with dsMAL (dashed line). Statistical analyzes were performed using the Student's t test (experimental versus their respective controls, dsMal). Data shown are mean ± S.E.,*$p<0.05$; *** $p<0.001$ (n=12-14).

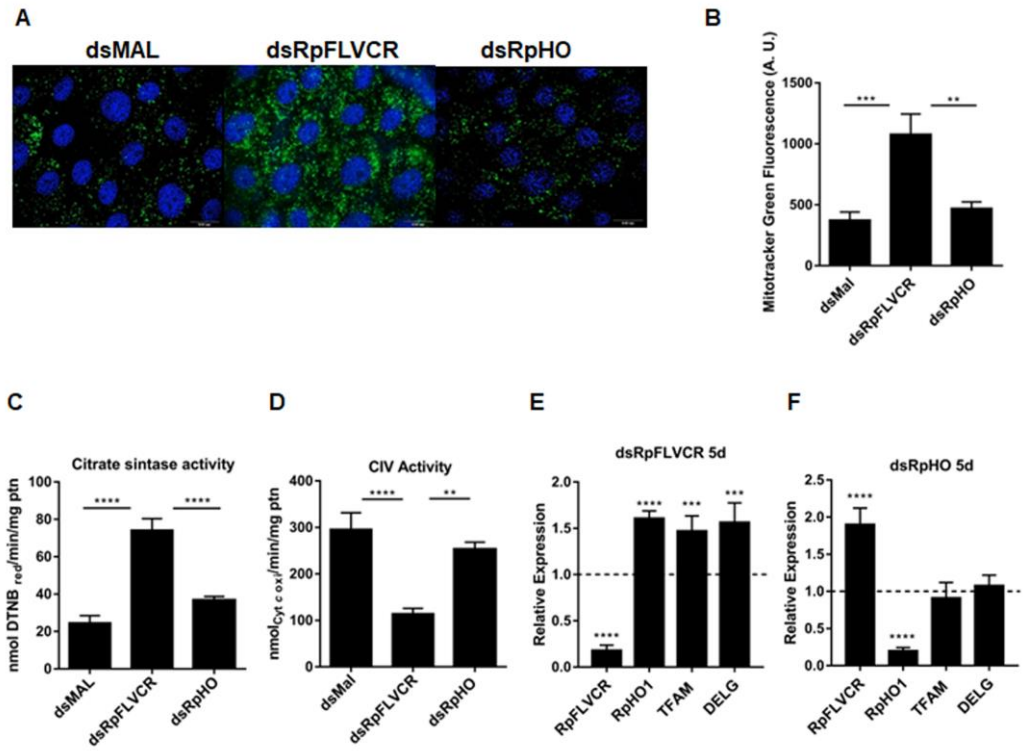
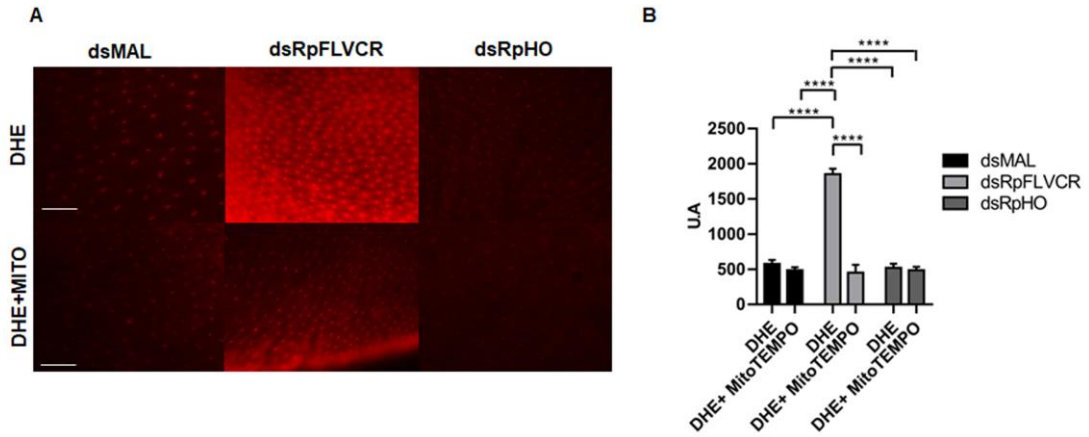


Figure 4: Knockdown of FLVCR increases mitochondrial biogenesis in the midgut. (A) Unfed *R.prolixus* females were injected with dsMal, dsRpFLVCR or dsRpHO; 3 days after injection they were fed on blood. 5 days after blood meal the insects were incubated with Mitotracker Green and DAPI as described in the Experimental section. Scale represents 20 µm. All pictures were taken with exposure time 260ms. The images are representative of total of 18 intestines analyzed. (B) Quantification of MitoGreen fluorescence using Olympus quantification software.**P≤ 0,01; *** P≤ 0,001 One-way ANOVA with Tukey´s post-test. (C) Mitochondrial content of the midgut was also determined by measuring the activity of citrate synthase on silenced females as described in methods section. Data shown are mean ± S.E.****P<0,0001. One-way ANOVA with Tukey's post test (n=7). (D) Activity of cytochrome *c* oxidase was measured using a Shimadzu spectrophotometer UV-visible 2450. Data shown are mean ± S.E. ** P≤ 0,01; ****P≤ 0.0001, One-way ANOVA with Tukey's post-test (n=8).(E) dsRpFLVCR and (F) dsRpHO injected- females had their midgut dissected 5 days after feeding. Transcript levels of genes that controls mitochondrial mass were measured by quantitative RT-PCR. The result was

normalized to animals injected with dsMAL (dashed line). Statistical analyzes were performed using the Student's t test (experimental versus their respective controls, dsMal). Data shown are mean ± S.E.*** P≤ 0,001 ; ****P≤ 0.0001.(n= 8-11) TFAM; mitochondrial transcription factor A; DELG; CG6338/Drosophila Ets like gene.

1



2

3 **Figure 5: Knockdown of FLVCR promotes a imbalance of mitochondrial**
4 **redox state**. (A) Anterior Midguts (AM) from FLVCR and HO-silenced females
5 were incubated with a 50µM solution of oxidant-sensitive fluorophore DHE or
6 the tissues were pre incubated with 50uM MitoTEMPO, a selective
7 mitochondrial antioxidant, prior to DHE incubation. dsMal-injected females were
8 used as a control. DHE oxidation in the AMs were analyzed by Fluorescence
9 Microscopy (Zeiss Axio Observer Microscope) using a 20× lens with 80 ms
10 exposure time for all conditions. The images are representative of a total of 10
11 midguts analyzed. Scale bar 2µm. (B) Graph represents the quantification of
12 fluorescence using Zeiss Axio Observer Quantification software. Data shown
13 are mean ± S.E.****P ≤ 0.0001 (One- way ANOVA followed by Tukey's test).

14