Supplementary Materials

Supplementary Text

Methods

Metabolomics

Midguts from mosquitoes were collected in lysis buffer and homogenized. The samples were then centrifuged and extracted following previously published procotols[76]. In brief, 30 µl aliquots were extracted by 1 ml of degassed acetonitrile: isopropanol: water (3:3:2, v/v/v) at -20°C, centrifuged and decanted with subsequent evaporation of the solvent to complete dryness. A clean-up step with acetonitrile/water (1:1) removed membrane lipids and triglycerides. The cleaned extract was aliquoted into two equal portions and the supernatant was dried down again. Internal standard C8-C30 fatty acid methyl esters (FAMEs) were added and the sample was derivatized by methoxyamine hydrochloride in pyridine and subsequently by N-methyl-N-trimethylsilyltrifluoroacetamide for trimethylsilylation of acidic protons. Data were acquired using the following chromatographic parameters, with more details to be found in previously published work[76]: (1) Column: Restek corporation rtx5Sil-MS (30 m length x 0.25 mm internal diameter with 0.25 µm film made of 95% dimethly/5%diphenylpolysiloxane); (2) Mobile phase: Helium; Column temperature: 50-330°C; Flow-rate: 1 mL min^{-1}; (3) Injection volume: 0.5 µL; (4) Injection: 25 splitless time into a multi-baffled glass liner; (5) Injection temperature: 50°C ramped to 250°C by 12°C s-1; (6) Gradient: 50°C for 1 min, then ramped at 20°C min-1 to 330°C, held constant for 5 min. Mass spectrometry parameters were used as follows. A Leco Pegasus IV mass spectrometer was used with unit mass resolution at 17 spectra s-1 from 80-500 Da at -70 eV ionization energy and 1800 V detector voltage with a 230°C transfer line and a 250°C ion source. Raw data files were preprocessed directly after data acquisition and stored as ChromaTOF-specific *.peg files, as generic *.txt result files and additionally as generic ANDI MS *.cdf files. ChromaTOF vs. 2.32 was used for data preprocessing without smoothing, 3 s peak width, baseline subtraction just above the noise level, and automatic mass spectral deconvolution and peak detection at signal/noise levels of 5:1 throughout the chromatogram. Apex masses were reported for use in the BinBase algorithm. Result *.txt files were exported to a data server with absolute spectra intensities and further processed by a filtering algorithm implemented in the metabolomics BinBase database. The BinBase algorithm used the settings: validity of chromatogram (<10 peaks with intensity >10^7 counts s-1), unbiased retention index marker detection (MS similarity>800, validity of intensity range for high m/z marker ions), and retention index calculation by 5th order polynomial regression. Spectra are cut to 5% base peak abundance and matched to database entries from most to least abundant spectra using the

following matching filters: retention index window ±2,000 units (equivalent to about ±2 s retention time), validation of unique ions and apex masses (unique ion must be included in apexing masses and present at >3% of base peak abundance), mass spectrum similarity must fit criteria dependent on peak purity and signal/noise ratios and a final isomer filter. Failed spectra were automatically entered as new database entries if s/n >25, purity <1.0 and presence in the biological study design class was >80%. All thresholds reflected settings for ChromaTOF vs. 2.32. Quantification was reported as peak height using the unique ion as default, unless a different quantification ion is manually set in the BinBase administration software BinView. A quantification report table was produced for all database entries that are positively detected in more than 10% of the samples of a study design class (as defined in the miniX database) for unidentified metabolites. A subsequent post-processing module was employed to automatically replace missing values from the *.cdf files. Replaced values were labeled as 'low confidence' by color coding, and for each metabolite, the number of high-confidence peak detections was recorded as well as the ratio of the average height of replaced values to high-confidence peak detections. These ratios and numbers were used for manual curation of automatic report data sets to data sets released for submission. Metabolites were identified by matching the ion chromatographic retention index, accurate mass, and mass spectral fragmentation signatures with reference library entries created from authentic standard metabolites under the identical analytical procedure as the experimental samples.

Results

Confirmation of inhibitor efficiency

A significant correlation was obtained with the identified metabolites from midguts of mosquitoes supplemented with either 4H-PG or HA (Pearson's $p < 0.05$; **Fig. S1**). This correlation indicated that the amino acid, fatty acid and derivatives, and glycolytic intermediate profiles followed similar trends with either treatment. In terms of NTs, treatment with 4H-PG alone resulted in higher content of excitatory NTs, namely Asp (1.2-fold), 5MT (2-fold), and *N*-acetyl-aspartate (1.5-fold, metabolite from the excitatory neuropeptide N-acetyl-aspartyl glutamate) relative to treatment with HA alone at 24 hours.

To ascertain whether 4H-PG was inhibiting CPT1, we analyzed metabolites that were detected and identified using GC-MS/MS from *A. stephensi* midguts at 24 hours after provision of 4H-PG in water-soaked pads. The values were normalized to the average of controls and the results were expressed as LOG2 values **(Fig. S2)**. The cut-off values were considered as either >0.3 or < -0.3 representing metabolites with concentrations higher or lower than controls, respectively. With inhibition of CPT1, decreased mitochondrial beta-oxidation of carnitine-dependent fatty acids (C12-C18) with increased fatty acid esterification (increase in fat deposits) was expected. Consistent with this expectation, a significant decrease in the content of midgut free fatty acids was observed interpreted as a shift from fatty acid oxidation (decrease in free fatty acids) to triacylglycerol synthesis (fatty acid esterification; **Fig. S2**).

To verify whether heptelidic acid (HA) was inhibiting glycolysis at the level of GAPDH activity **(Fig. S2)**, we performed metabolomic analyses of midguts from *A. stephensi* at 24 hours after provision of HA in water-soaked pads. From the metabolites detected, those located upstream from GAPDH were higher in HA-exposed midguts than controls (i.e., glucose and glucose-6-phosphate; in bold; **Fig. S3**) and those located downstream from GAPDH were lower (i.e.,

phosphoenolpyruvate and lactic acid). Assuming that the ratio of lactate-to-glucose is reflective of the activity glycolytic pathway (since pyruvate was undetectable), this ratio was 13-fold lower in HA-exposed midguts versus controls suggesting that glycolytic flux was indeed halted with HA. Significant decreases in free fatty acids (**Fig. S3**) pointed at a shift favoring midgut fatty acid oxidation to generate energy (in the absence of an operational glycolytic pathway).

Figures

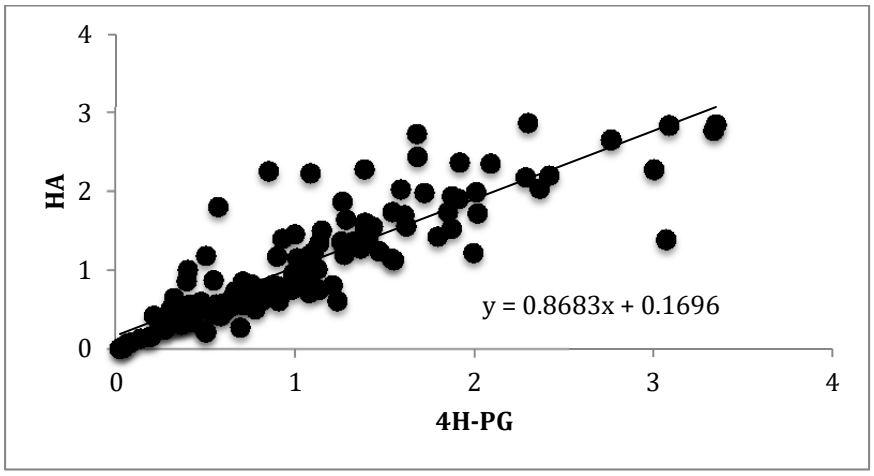


Fig. S1. Analysis of metabolites from midguts treated with metabolic inhibitors (4-hydroxy-L-phenylglycine, 4H-PG; heptelidic acid, HA) alone for 24 hours. A significant correlation was obtained from metabolites identified in midguts treated with each inhibitor. Levels in inhibitor treated groups were normalized to control.

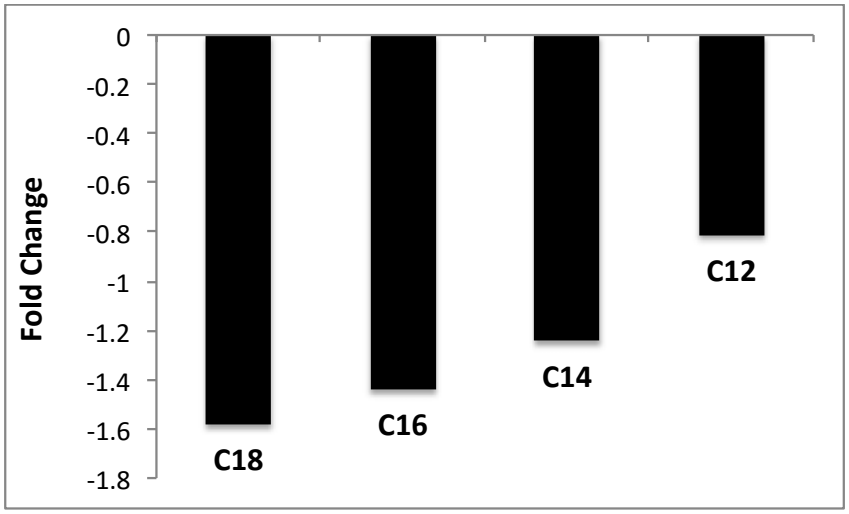
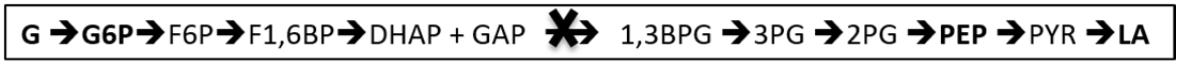


Figure S2. Decreases in midgut free fatty acids from *A. stephensi* treated with 4H-PG
Midguts were dissected at 24 hours after provision of 1 mM 4H-PG in water-soaked pads and subjected to metabolomics analyses. Fatty acids relevant to mitochondrial beta-oxidation are

shown as fold changes relative to control values. Statistically significant changes were considered at >0.3 or <-0.3.



Compound	Fold change
glucose	1.16
glucose-6-phosphate	0.64
phosphoenolpyruvate	-1.50
lactic acid	-2.56

Figure S3. Inhibition of GAPDH by HA
Simplified glycolytic pathway with intermediates, showing those upstream from GAPDH and downstream from this enzyme; compounds in bold were detected by metabolomics. Fold changes relative to controls of those identified are shown to the left. Metabolites upstream of GAPDH accumulated in the midgut indicating reduced processing due to inhibition of GAPDH activity. Accordingly, compounds downstream of GAPDH were reduced due to an inability of GAPDH to convert upstream compounds.

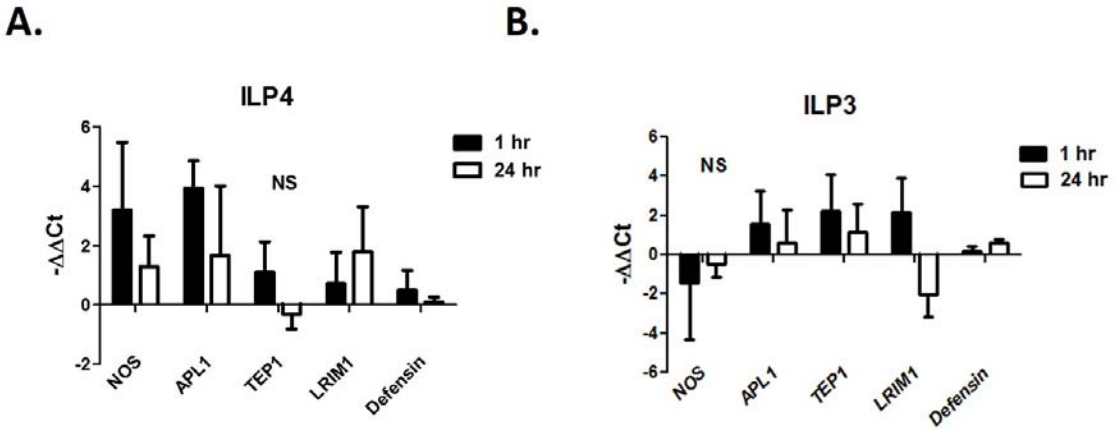


Figure S4. Effects of metabolic inhibitors on NF-kB mediated immune gene expression in response to *P. falciparum*. Cohorts of *A. stephensi* were pre-treated with either **(A)** CPT1 inhibitor (1 mM 4H-PG) or **(B)** GAPDH inhibitor (10 µM HA) in water-soaked cotton pads for 24 hours to inhibit beta-oxidation and glycolysis, respectively. Mosquitoes were then provided a meal of human RBCs with *Pf*Ps and supplemented with **(A)** 170 pM ILP3, **(B)** 170 pM ILP4 or an equivalent volume of diluent. Midguts were dissected at 1 and 24 hours post-feeding for RNA extraction and qRT-PCR analysis of *NOS*, *Defensin*, *LRIM1*, *APL1*, or *TEP1* gene expression. Data from replicates with three separate cohorts of *A. stephensi* of 150-180 mosquitoes each are represented as mean -ΔΔCt values ± SEM on a Log2 scale (1 Ct value = 2-fold change, controls not pre-treated with inhibitors set at 0). Data were analyzed by t-test after normalization to a linear scale in order to determine differences between inhibitor pre-treated and control groups (set at 0). *P-values were deemed significant when P≤0.05. NS=not significant.

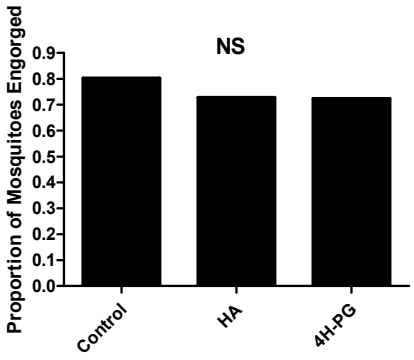


Figure S5. Effects of metabolic inhibitors on engorgement success in *A. stephensi.*
Mosquitoes were pre-treated with GAPDH inhibitor (10 μM HA) or CPTI inhibitor (1 mM 4H-PG) in water-soaked cotton pads for 24 hours. Subsequently, mosquitoes were offered a meal of uninfected human RBCs and the proportion of mosquitoes that fully engorged within 15 minutes were counted. The experiment was performed with 70-100 mosquitoes for each group. Data were analyzed by chi-square test to determine significant differences between inhibitor and water-treated controls. NS= not significant.