TITLE

Bat power-metabolic profiling of the Egyptian fruit bat *Rousettus aegyptiacus* reveals distinctive cardiac adaptations

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ABSTRACT

Aim

The present study aimed to elucidate which pathways contribute to cardiometabolic adaptation in Egyptian fruit bats.

Methods

Utilising cardiac tissues from Egyptian fruit bats (*Rousettus aegyptiacus*) and C57BL/6J mice, we combined liquid chromatography-mass spectrometry metabolic profiling, non-targeted ^{1}H NMR spectroscopy, and in silico computational modelling using the genome-scale mammalian network CardioNet.

Main Findings

Our analyses revealed that bat hearts exhibit a distinct metabolic profile characterised by depleted glycogen reserves and increased reliance on lipid oxidation to meet energy demands. Notably, bat hearts displayed elevated fluxes in oxidative phosphorylation, β-oxidation of long-chain fatty acids, and the Krebs cycle, alongside reduced amino acid catabolism. These findings suggest that bats have evolved unique metabolic strategies to support the high-energy demands of flight, maintaining cardiac function without succumbing to pathological remodelling.

Conclusions

This study provides the first comprehensive insight into the metabolic adaptations in the cardiac tissue of a bat species, contributing to our understanding of how these mammals endure extreme physiological stresses. Integrating metabolomics with computational modelling offers a powerful approach to studying metabolic adaptations in non-model species, potentially informing therapeutic strategies for human cardiac conditions.

1. INTRODUCTION

Metabolic adaptation is a molecular mechanism that enables mammals to occupy a wide range of ecological niches and extend the limits of their physiological capacity. Bats stand out among mammals for their hypoxia tolerance, DNA repair, and anti-inflammatory mechanisms.[1-3] These traits allow bats to roost in caves with lower-than-ambient oxygen availability. Nonetheless, bats are among the longest-lived small mammalian species, with lifespans greater than those of non-flying eutherian mammals of comparable body size. Bats are the only mammal with true, self-powered flight, during which heart rate and cardiac workload are drastically increased for prolonged periods.[2,4] Given these distinct traits, bats are considered model species for studying the molecular basis of adaptation, extended lifespan, and resistance to pathologic cardiac remodeling.[5]

Transcriptomic analyses of bat hearts have uncovered an alternative splice form of the cardiac troponin I gene (*TNNI3*)[4], which accelerates diastolic relaxation and supports elevated heart rates without adrenergic stimulation.[4] These data imply that bats overcome persistent exposure to hemodynamic and oxygen stress through improved diastolic performance and metabolic coupling, avoiding the onset of pathological cardiac remodelling and heart failure.[2] The mechanism by which metabolic adaptation supports extreme variations in cardiac workload in bats is not well understood.

In most countries bat species are endangered, protected, and only small colonies can survive in captivity. Therefore, sampling for experimental validation of proposed molecular mechanisms is prohibited and limited to biopsies. To bridge this gap, transgenic mice have been developed[6] to translate non-model species such as naked mole rats, allowing comparative analysis to reveal patterns of cardiac adaptation[3,7,8]. However, similar models are missing for bats. Integrating *ex vivo* experimental data with computational modelling offers an effective approach to minimise reliance on in vivo animal experiments. Utilising bat tissues, published physiological data, and advanced computer simulations enables more accurate and ethical outcomes in biomedical studies. The mammalian network of cardiac metabolism, CardioNet, allows the visualisation of metabolic topologies and the prediction of metabolic fluxes to reveal species-specific cardiometabolic profiles. We hypothesised that metabolic profiles in bats differ from mice in maintaining high metabolic rates during flight. To test this hypothesis, we used cardiac tissue from captive Egyptian fruit bats (*Rousettus aegyptiacus*) and compared their metabolic profiles to C57/BL6J mice via ^{1}H NMR and mass spectrometry-based metabolomics combined with computational CardioNet modelling. Both bats and mice have a comparable resting heart rate (450bpm), which increases significantly during periods of activity (e.g., flight in bats or running in mice) to meet the increased oxygen and energy demands[2,9]. Our flux predictions reveal the consequences of high-energy demands on lipid pools in bats and identify preferred nutrient compositions to meet energy requirements. Finally, the modelling approach showed that lipid availability and cardiac glycogen stores influence the allocation of energy reserves in the heart.

2. MATERIAL AND METHODS

2.1. Animals.

Zoo colony of Egyptian fruit bat (*Rousettus aegyptiacus*). Heart tissue was obtained from captive adult Egyptian fruit bats (*Rousettus aegyptiacus*, n=4) at the Copenhagen Zoo in Denmark. Animals were euthanised by trained veterinarians in line with management decisions in the zoo to ensure the best welfare of the captive colony.

C57/Bl6 mice. 20-week-old male C57/Bl6 mice (n=4) were used in this study (Charles River, UK). Mice were housed in individually ventilated cages and maintained under controlled temperature (22 ± 2 °C) on a 12:12 h light-dark cycle with access to a standard chow diet (irradiated PicoLab® Mouse Diet 20 EXT, 5R58) and water *ad libitum*. Animal experiments were conducted by the UK Home Office Animals (Scientific Procedures) Act, 1986. Mice were sacrificed by phenobarbital I.P. injection, and hearts were rapidly excised and snap-frozen in liquid nitrogen.

2.2. Heart tissue collection.

Dissected hearts were stabilised in RNAlater, snap-frozen in liquid nitrogen, and stored at -80°C until further analysis. Metabolites were extracted from frozen tissue as previously described[3,4].

2.3. Metabolomics

2.3.1. ^{1}H Untargeted NMR spectroscopy.

Snap frozen and pulverised cardiac tissue samples (~45 mg) were subject to methanol/water/chloroform phase extraction and analysed using ^{1}H NMR high-resolution spectroscopy as previously described[7,10]. ^{1}H NMR spectra were acquired using a vertical-bore, ultra-shielded Bruker 14.1. Tesla (600 MHz) spectrometer with a BBO probe at 298K using the Bruker noesygppr1d pulse sequence. Acquisition parameters were 128 scans, 4 dummy scans, and 20.8 ppm sweep width, acquisition time of 2.6s, pre-scan delay of 4s, 90° flip angle, and experiment duration of 14.4 minutes per sample. TopSpin (version 4.0.5) software was used for data acquisition and metabolite quantification. FIDs were multiplied by a line broadening factor of 0.3 Hz and Fourier-transformed, phase and automatic baseline-correction were applied. Chemical shifts were referenced to the TSP signal. Metabolite peaks of interest were initially integrated automatically using a pre-written integration region text file and then manually adjusted where required. Assignment of metabolites to their respective peaks based on previously obtained in-house data, confirmed by chemical shift and using Chenomx NMR Profiler Version 8.1 (Chenomx, Canada). Peak areas were normalised to the total metabolite peak area. Glycogen was quantified using ^{1}H magnetic resonance, measuring the concentration of glucose monomers from normalised peaks. Being a large macromolecule, with possible differences in the mobility of glycosyl units, glycogen has been reported to be fully visible by MRS. The modified dual-phase Folch extraction method, used for separating aqueous and lipid metabolites, was not optimised for extracting glycogen. However, all samples underwent the same extraction procedure, allowing between treatment group comparison[7].

2.3.2. Targeted LC-MS/MS

Snap-frozen and pulverised cardiac tissue samples (~15 mg) were extracted using a buffer (50% Methanol, 30% Acetonitrile, 20% ultrapure water, and 50 ng/ml 1 mM HEPES solution) for LC-MS analysis. LC-MS analysis was performed using a Q Exactive Quadrupole-Orbitrap mass spectrometer coupled to a Vanquish UHPLC system (Thermo Fisher Scientific). The liquid chromatography system was fitted with a Sequant ZIC-pHILIC column (150 mm × 2.1 mm) and guard column (20 mm × 2.1 mm) from Merck Millipore (Germany), and the temperature was maintained at 35°C. The sample (3µL) was separated at a flow rate of 0.1 mL/min. The mobile phase comprised 10 mM ammonium bicarbonate and 0.15% ammonium hydroxide in water (solvent A), and acetonitrile (solvent B). A linear gradient was applied by increasing the concentration of A from 20 to 80% within 22 min and then maintained for 7 minutes. The mass spectrometer was operated in full MS and polarity switching mode, in the range of 70-1000m/z and resolution 70000. Major ESI source settings: spray voltage 3.5kv, capillary temperature 275°C, sheath gas 35, auxiliary gas 5, AGC target 3e6, and maximum injection time 200ms. The acquired spectra were analysed using XCalibur Qual Browser and XCalibur Quan Browser software (Thermo Scientific) for the targeted MS analysis.

2.4. CardioNet-based metabolic flux analysis.

In silico simulations were conducted using the metabolic network of the mammalian heart metabolism, CardioNet[11-13]. Mathematical modelling has previously been used to study the dynamics of cardiac metabolism in response to stress. Metabolite abundances from NMR and LC-MS/MS analysis were integrated as constraints for metabolite pools. The objective was to maximize ATP hydrolysis as a reflection of cardiac contraction (vATPase) that satisfies the metabolic constraints. We determined flux distributions (v_m) and estimated flux rate changes (v_{FC}) as described previously[10,11,14,15]. We simulated rapid flight, night and roost activity in bats using publicly available telemetry data of bat heart rates[9]. The demand for ATP per heartbeat was based on the transient ATP increase upon excitation[16]. Further, metabolites within the model were constrained based on our metabolomics analysis. The uptake of glucose, ketone bodies, amino acids and lipids was not restricted. The GUROBI LP solver was used to find the solution to the FBA problems[17].

2.5. Statistical Analysis.

Statistical analysis was conducted using GraphPad Prism software (v.10.40.1) and R studio (v.2024.09.0). Differences between groups were considered significant at $p<0.05$. Sample sizes were not predetermined based on statistical power calculations. Formal randomizations of mouse or bat experiments were not used. Data were tested for normal distribution and similar variance among treatments using the Shapiro-Wilk tests. Statistical significance was calculated by multiple unpaired t-tests and comparisons analysis using a false discovery rate < 5% by the two-step method of Benjamini, Krieger, and Yekutieli, 2-way ANOVA followed by multiple comparison analysis by Sidak.

3. RESULTS

We collected heart tissue from a zoo colony of the Egyptian fruit bat (*Rousettus aegyptiacus*) (**Figure 1A**) undergoing population management to determine the metabolic mechanisms underlying cardiac physiological adaptations in bats. Myocardial tissue was harvested post-euthanasia from adult bats (male and female) and compared to tissue obtained from C57/BL6 mice (male, 20 weeks old). To elucidate the metabolic profile of the bat heart, we used an integrated study approach combining untargeted high-resolution ^{1}H NMR spectroscopy-based metabolomics with targeted liquid chromatography mass-spectroscopy (LC-MS) metabolic profiling (Barts Cancer Institute Core Facility), and *in silico* CardioNet mammalian metabolic network flux balance analysis (**Figure 1A**).

Principal component analysis of metabolomics data revealed that Egyptian fruit bats have a distinct cardiometabolic profile compared to C57/BL6J mice (**Figure 1B**). Comparative analysis revealed that the abundance of 43 profiled metabolites significantly differed between bats and mice (**Figure 1C**). Notably, cardiac energy reserves were depleted in bat hearts, as evidenced by reduced creatine and undetectable glycogen (**Figure 1D**). In the heart, glycogen reserves are utilised during periods of high physical activity[18]. Once glycogen stores are depleted, lipid oxidation increases and becomes the sole energy-providing substrate. AMP signals a highly catabolic cellular state and allosterically inhibits phosphofructokinase in the glycolysis, which supports replenishing glycogen pools and the oxidation of lipids[15]. Consistent with these findings, we found increased AMP levels in bat hearts (3.5-fold vs. mouse, **Figure 1E**), while $NAD(P)^{+}$ redox equivalents were reduced. In the bat heart, glycolytic and Krebs cycle intermediates (**Figure 1F**) and amino acids (**Figure 1H**) were reduced compared to mice. Only constituents of lipid metabolism (**Figure 1G**) were universally increased (i.e., acetate, choline, phosphocholine, and carnitine), indicating an increased contribution of lipids to cardiac metabolism in bats. Correlation analysis of metabolic intermediates (**Supplementary Figure 1**) revealed co-regulation of ketogenic amino acids entering the Krebs cycle through acetyl-CoA. Increased ketone body metabolism spares protein degradation and further supports lipid oxidation. Together, our metabolomics findings suggest metabolic adaptation in the bats driven by the depletion of glucose-derived energy reserves followed by increased oxidation of lipids to meet cardiac energy demands.

We next elucidated cardiac metabolic profiles mechanistically by integrating metabolomics data into the mammalian network of cardiac metabolism, CardioNet[13]. Using this approach, we conducted unbiased computational modelling through flux balance analysis (FBA) to predict flux distributions in each species. Our systems biology approach revealed that bat hearts show a manifold increase in cardiac metabolic flux of oxidative phosphorylation, β-oxidation of long-chain fatty acids, glutamate, pyruvate, Krebs cycle (α-ketoglutarate), and total adenine nucleotide pool (AMP, ADP, ATP; **Figure 1A**). Furthermore, bat hearts were characterised by the markedly elevated flux of fructose, ketone body metabolism, and reduction in amino acid catabolic flux exchange (**Figure 1B** and **1C**). Our data indicate that bat hearts, like bat skeletal muscles[3], have evolved unique metabolic adaptations characterised by enhanced metabolic fluxes through oxidative metabolism. Thus, bats appear to have evolved a cardiovascular system with the superior ability to adapt their metabolism flux to match cardiac workload and output during prolonged stress.

Finally, we compared predicted flux distributions from different activity states in bats, where experimentally determined heart rates as a function of ATP demands from bats during night, roost, and flight activities[9] were integrated into CardioNet simulations. PCA analysis revealed that predicted metabolic flux

distributions differ based on the simulated activity status (**Supplementary Figure 2B**). Predicted myocardial oxygen consumption increased with activity status (**Figure 2C**), with flight showing the highest consumption rate, followed by night and roost activity. These differences were also reflected in the consumption of energy-providing nutrients. The glucose utilisation is predicted to be low in all three activity states (**Figure 2D**). The uptake and oxidation of linoleate (FA18:2) is highest during flight, while β-hydroxybutyrate contribution increases significantly during roost and night activity. This difference was also reflected at the level of the electron transport chain fluxes (**Figure 2E**), where complex I and V fluxes increase significantly during flight and are lowest during roost activity. Thus, fatty acids are major energy-providing substrates, and ketone bodies further complement ATP provision during roost and night activities.

4. DISCUSSION

One of the fundamental challenges in cardiac metabolism is linking metabolic profiles to structural changes and disease stages. Analysis of metabolic states can reveal metabolic vulnerabilities and adaptations that are potential therapeutic targets. Comparative analysis of mammals adapted to extreme environments provides insights into metabolic drivers for cardiac adaptation. The current work shows that bats achieve remarkable ATP demands by optimizing the oxidation of long-chain fatty acids and ketone bodies independent of glucose.

Compared to mice, bats expend double the amount of metabolic energy over their lifetimes while accruing remarkably little oxidative stress. Bats possess mitochondria that produce significantly less H_2O_2 and consume less O_2[1]. Computational modelling using flux analysis corroborates these previous findings and reveals an increased contribution of long-chain fatty acids and amino acid metabolism compared to mice. These findings are consistent with the last metabolomic assessments of pectoral muscle and liver tissue from 49 neotropical bat species[3], which identified convergent amino acid substitutions in the otherwise evolutionarily conserved aldolase B—the rate-limiting step regulator in fructolysis and glycolysis—and the mitochondrial pyruvate dehydrogenase. Additional tracer-based studies may complement these in silico modelling analyses to better elucidate nutrient preferences.

Simulation of activity states revealed that bats shift from predominately oxidizing fatty acids (FA18:2) to ketone bodies (β-hydroxybutyrate) during lower activity states at night and during roosting. Interestingly, ketone body and branched-chain amino acid oxidation exceed glucose oxidation *in silico*, which requires further experimental validation. Our findings imply an alternative role of glucose in addition to the provision of ATP. Given that bats comprise >1400 species and include lineages with derived traits such as torpor and viral tolerance, it is likely that future studies will uncover further metabolic adaptations in this remarkable group.

CRediT authorship contribution statement

A.K.: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **F.C.:** Writing – review & editing, Investigation, Formal analysis. **R.D.:** Writing – review & editing, Investigation, Formal analysis. **K.K.:** Writing – review & editing, Investigation, Formal analysis. **M.Y.:** Writing – review & editing, Investigation, Formal analysis. **S.J.R.:** Writing – review & editing, Investigation, Formal analysis. **D.A.:** Writing – review & editing, Investigation, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualisation.

Data statement

All the data acquired are presented in the figures

Declaration of competing interest

The authors declare there are no financial interests/personal relationships that may be considered as potential competing interests.

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Figures and Figure Legends

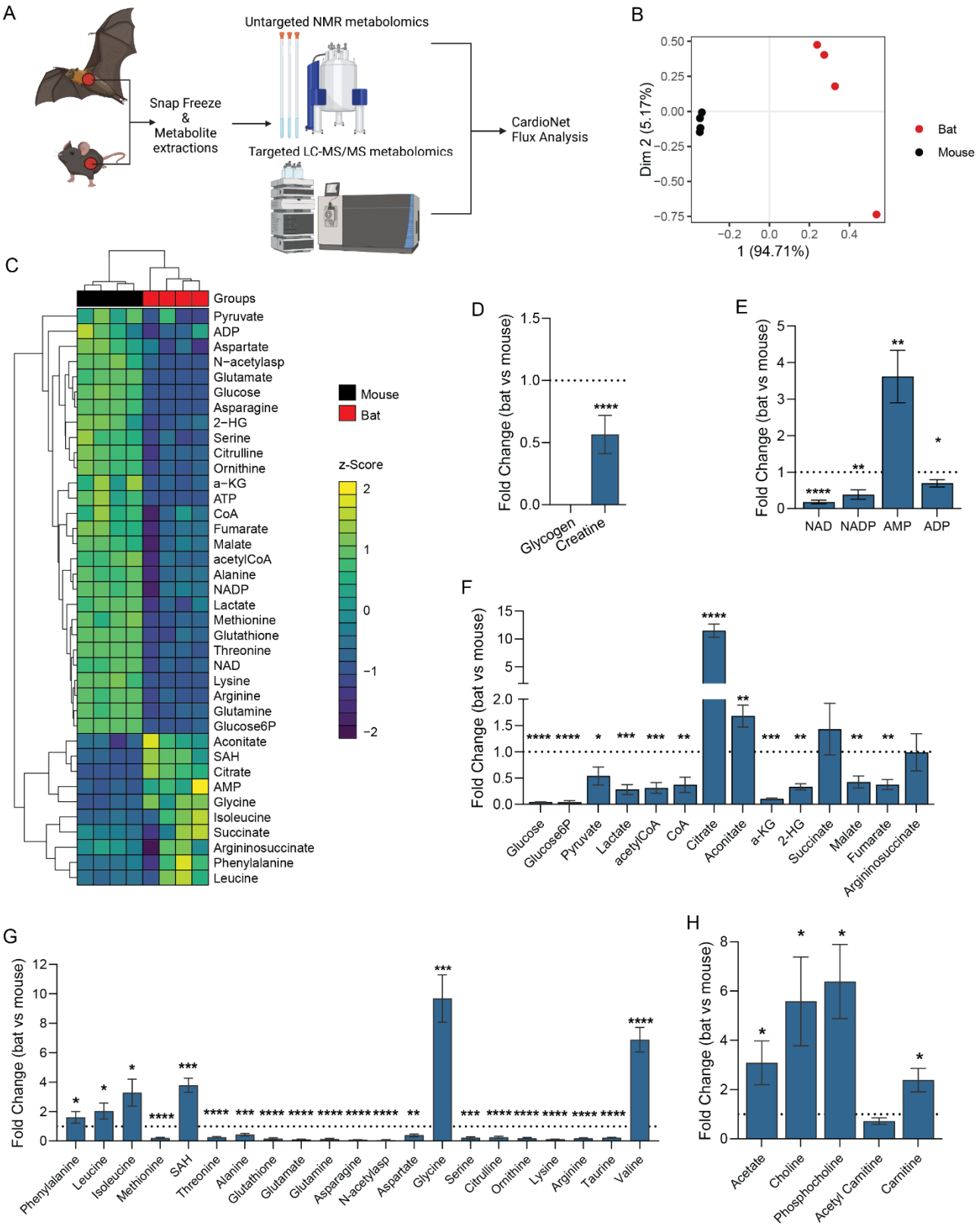


Figure 1. Cardiac metabolism varies between Egyptian fruit bats and C57BL/6J mice.

(**A**) Schematic summary of the study protocol. n=4 bats (n=2 males, forearm length=91.60±2.50 mm, n=2 females, forearm length=87.90±1.85 mm) and n=4 C57/BL6 mice (Charles River, UK, male, 6 weeks). (**B-C**) Partial Least Squares Discriminant Analysis (PLS-DA) (**B**) and heatmap of unsupervised hierarchical cluster analysis (**C**) of the cardiac metabolite concentrations determined by LC-MS and high-resolution ^{1}H nuclear magnetic resonance spectroscopy showing distinct profile of bat and C57/BL6 mouse. (**D-H**) Fold change differences of myocardial intermediates within major metabolic pathways (glucose metabolism, lipid

metabolism, Krebs cycle), redox equivalents and energy-providing substrates. Fold changes are expressed as comparisons between bats and C57/BL6 mouse. The normality of data distribution was examined using Shapiro–Wilk's normality test. Comparison between two groups was performed by Student's t-test (Gaussian data distribution) or Mann-Whitney U test when data was not normally distributed, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

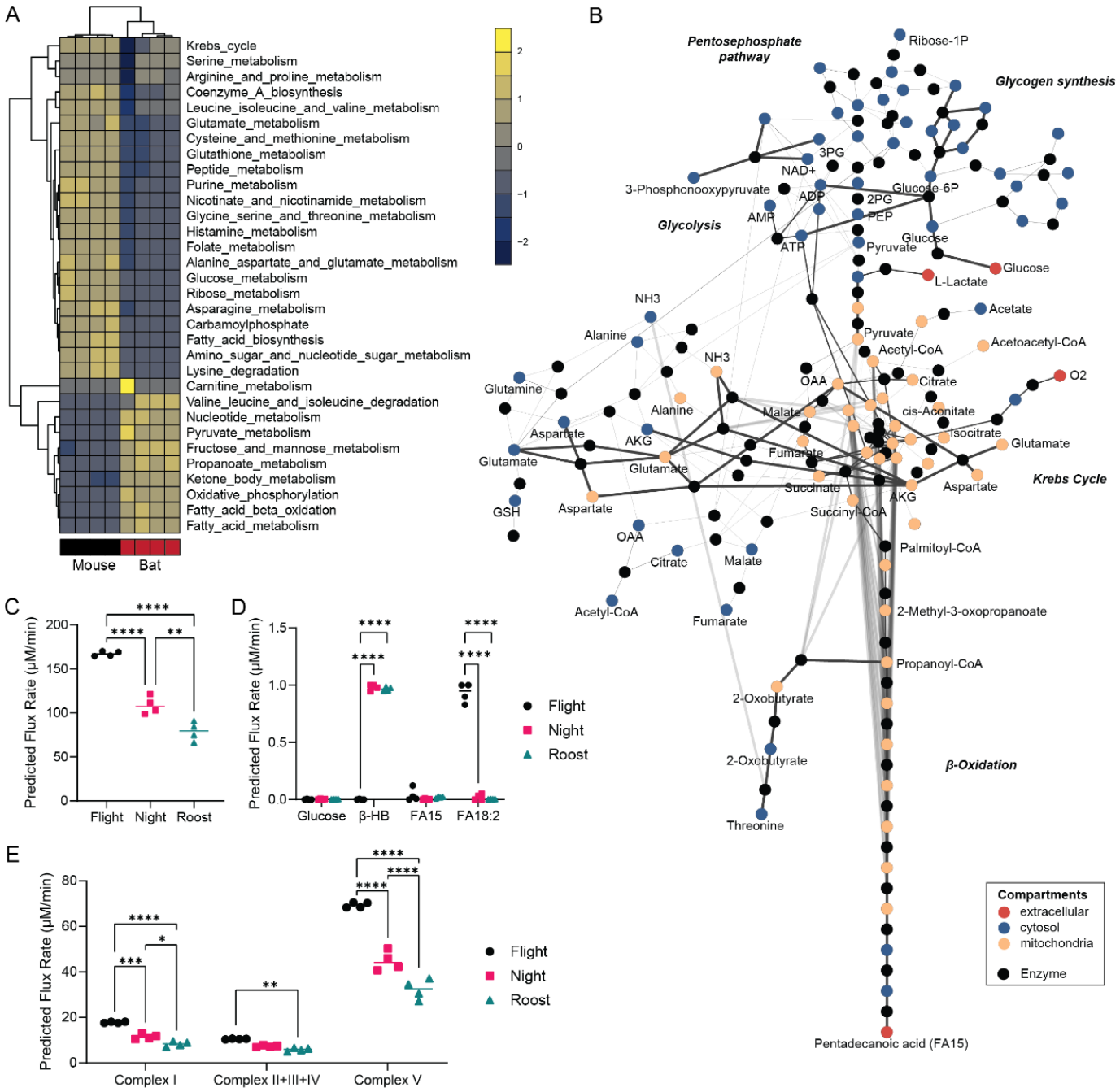
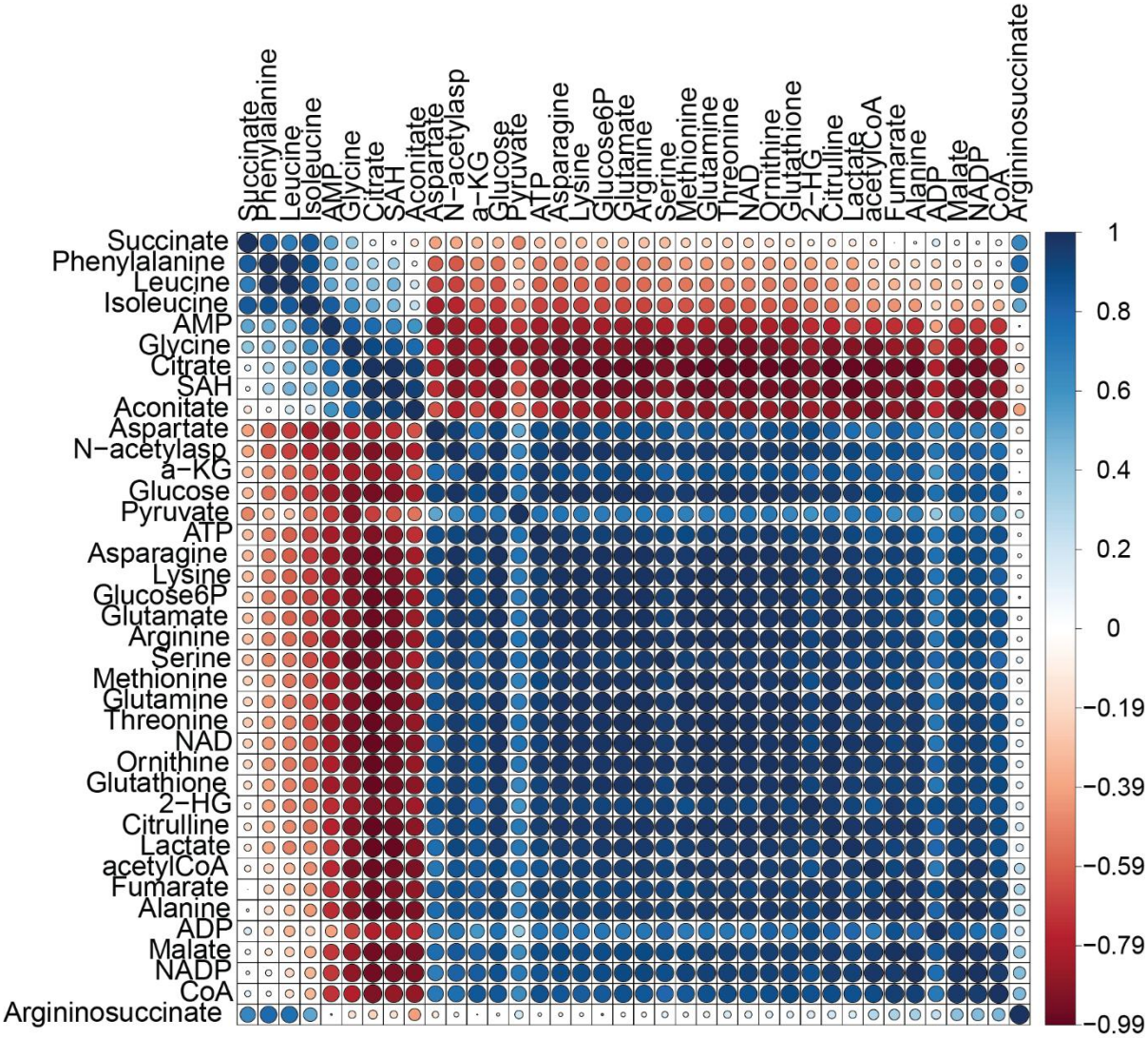


Figure 2. CardioNet analysis reveals increased lipid oxidation flux in bats.

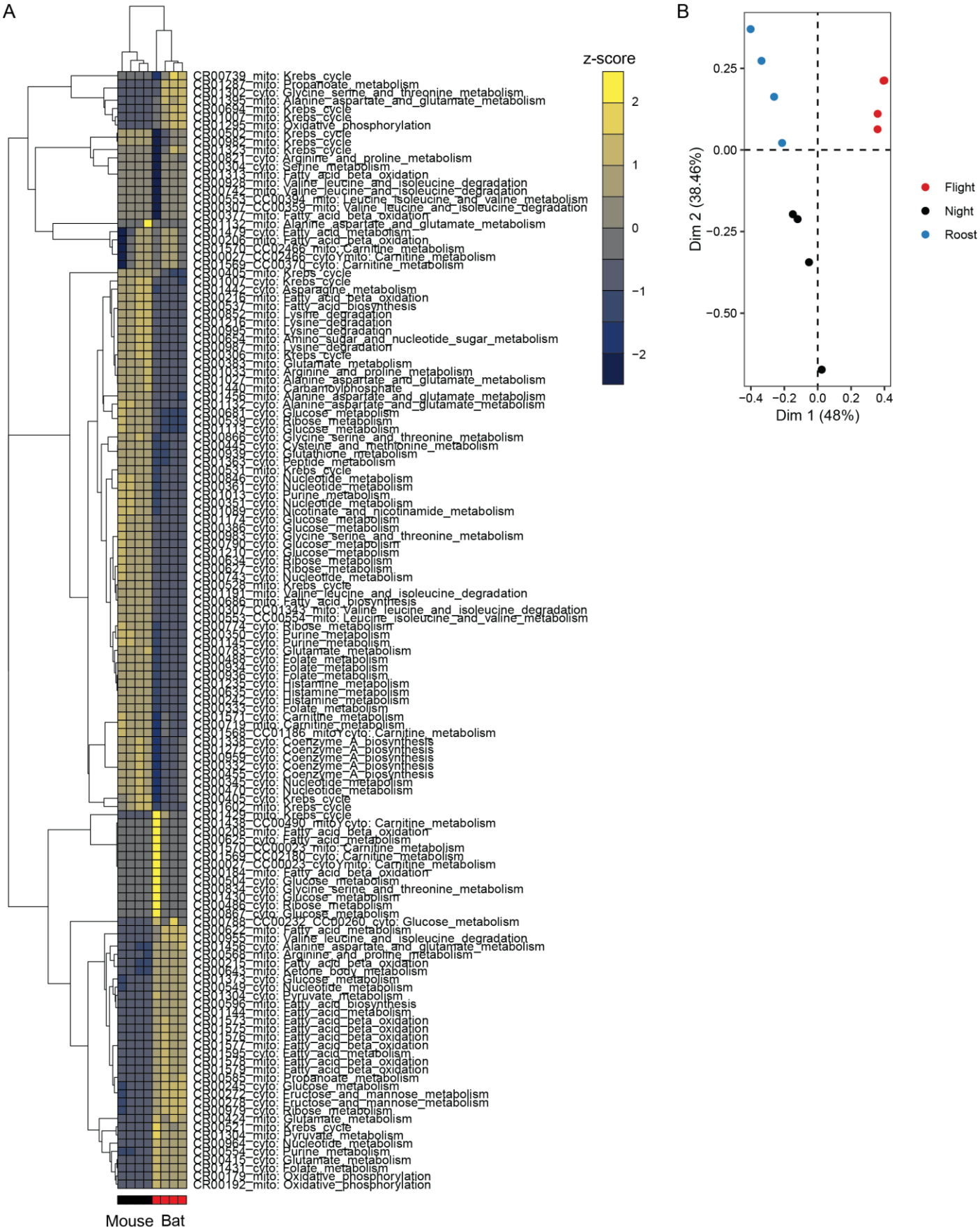
(**A**) Unsupervised hierarchical clustering of estimated z-scored flux rate changes reveals metabolic adaptation in bats vs mice. Z-scores were calculated to visualise how many standard deviations an estimated flux rate is away from the mean across all experimental groups. The z-score describes the distance from the mean for a given flux rate as a function of the standard deviation. The colour scale indicates the degree to which estimated flux rate changes are predicted to be respectively lower or higher bat vs mouse. (**B**) CardioNet *in silico* metabolic >5-fold flux changes bat versus C57/BL6. The graph denotes the estimated flux distribution. The coloured nodes represent metabolites assigned to five compartments: extracellular space, cytosol, mitochondria, microsome, and lysosome. The black nodes indicate reactions; two reactions are linked by a directed edge, indicating the reaction flux. The line thickness of each edge is proportional to the predicted flux rate change. (**C**) Predicted myocardial oxygen consumption in simulations of bat flight, night activity and roost activity (n=4/group). (**D**) Predicted substrate uptakes for glucose, β-hydroxybutrate (β-HB) and fatty acids (FA15, FA18:2) in simulations

of bat flight, night activity and roost activity (n=4/group). (**E**) Predicted flux rates for oxidative phosphorylation and electron transport chain in simulations of bat flight, night activity and roost activity (n=4/group).

Supplementary Figures



Supplementary Figure 1. Correlation analysis of bat metabolome. Metabolite abundances from NMR- and LC-MS/MS-based metabolomics were analysed using Spearman correlation to reveal interactions between intermediates. Positively and negatively correlating intermediates are indicated by blue and red colour coding, respectively. Circle sizes indicate calculated absolute spearman coefficient ranging from 0 to 1.



Supplementary Figure 2. *In silico* modelling of bat metabolism. (**A**) Unsupervised hierarchical cluster analysis and heatmap of significantly altered metabolic fluxes in bat and mouse simulations using CardioNet. Flux rates were z-score normalised. n=4/species. (**B**) Principal component analysis of simulated activity states: flight, night and roost.

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