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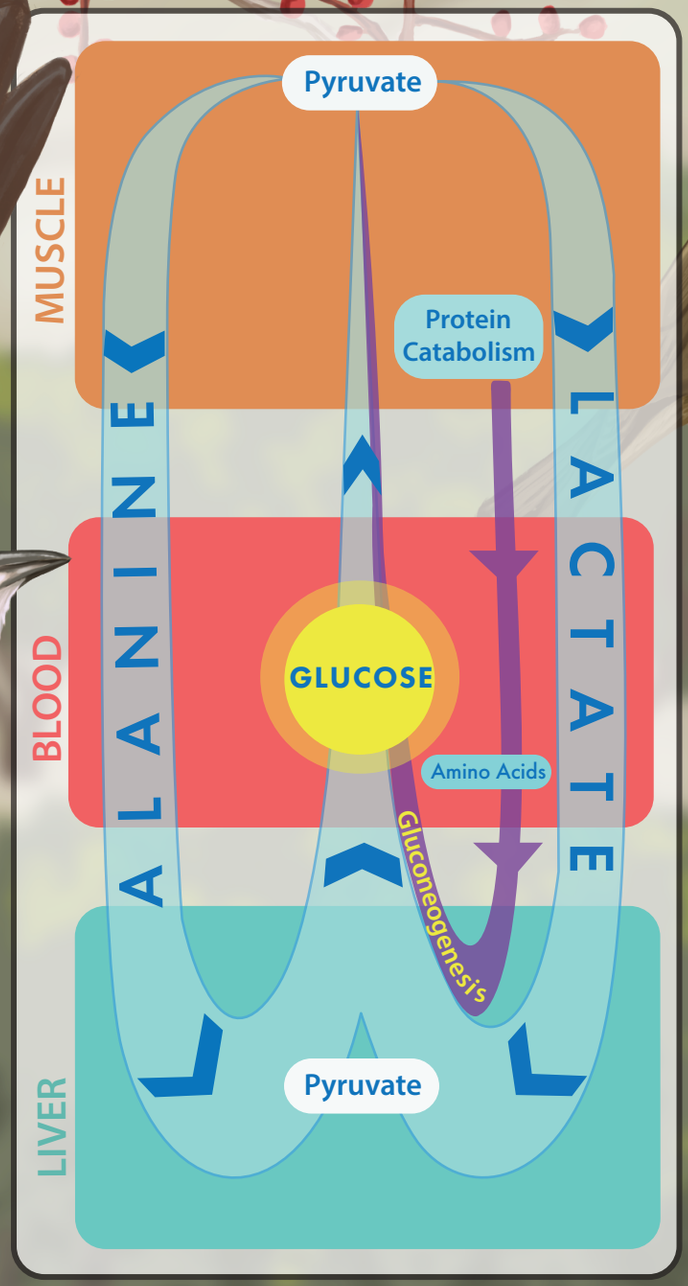
Birds landing at low-quality stopover sites depart soon after arrival.

Birds landing at high-quality stopover sites recover and gain body mass.

During migratory recovery, birds cycle glucose, derived from protein catabolism.



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Comparative analysis of the plasma metabolome of migrating passerines during stopover: Novel insights into flight metabolism

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Abstract

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During long distance migration, many birds may experience periods of either prolonged fasting, during endurance flights, or extensive feeding during stopovers. It was previously shown that habitat selection during stopover can largely affect the migration outcome of an individual. Despite decades of research of the avian metabolism during stopover and migration, many questions have remained unanswered, as such research mainly focused on targeted metabolites and fat metabolism. Here, we examined the plasma-metabolome of migrating passerines prior to their crossing the Sahara Desert. Birds were sampled at two sites populated by Pistacia trees, bearing fat-rich fruits, and at an additional site dominated by blooming Eucalyptus trees. The blood samples were analyzed using both GC-MS and LC-MS, using an untargeted approach. We found that birds from one of the sites had a distinguish metabolic profile, suggesting recent landing. Examination of metabolic pathways activated during stopovers indicated a crucial role for cycling glucose through the Cori and Cahill cycles in resting and recovery processes. This novel perspective, conducted on free-ranging birds, suggests the evolution of avian insulin resistance due to factors such as endurance exercise, fasting, and a preference for fatty acid oxidation during migration, akin to cell trauma recovery. Additionally, we investigated inter-site variations in birds' metabolic profiles. Significant variations were observed in both polar and lipophilic metabolites among the sites. Differences in polar metabolites were primarily attributed to variations in the physiological state of the birds between sites, while distinctions in the lipophilic profiles of rested birds were linked to variations in their primary food sources. This study underscores the challenge of interpreting commonly used indicators for assessing migrating birds' physiological states and site quality, which are predominantly derived from lipid metabolism, in complex ecological systems.

Introduction

Animal migration – one of nature's most visible and widespread phenomena (Wilcove and Wikelski 2008) – has evolved independently several in varying taxa (Aidley 1981). Migratory behavior is widely common in the avian taxon, with approximately half of the species performing some type of migratory movements (Berthold 1996). Migratory birds alternate between two extreme physiological states, fasting during the long-distance endurance flights and resting or extensively feeding during stopovers (McWilliams and Karasov 2005). Hence, selecting a proper stopover site is crucial for long-distance migrants as low fuel deposition rates can extend their total migration period and affect their fitness (Gómez et al. 2017, Domer et al. 2021). We present an in-depth comparative analysis of the untargeted metabolomic profiling of wild migratory passerines sampled in the eastern Mediterranean region during autumn, along one of the most important flyways in the old world. Previous targeted metabolic studies on wild and captive migratory birds have provided important insights into flight metabolism modalities, including fuel utilization (Jenni and Jenni-Eiermann 1998, Jenni-Eiermann et al. 2002, Smith et al. 2007), protein catabolism (Robin et al. 1987, Smith et al. 2007), and oxidative damage repair of flight muscles (Costantini et al. 2007). While these studies have laid the foundations for the metabolic migration framework, they considered only a few targeted metabolites (Jenni-Eiermann and Jenni 1991, Jenni and Jenni-Eiermann 1998, Jenni-Eiermann et al. 2002, Guglielmo et al. 2005, Seaman et al. 2005) while mainly focusing on lipid metabolism. Flight metabolism comprises many inter-dependent pathways and modalities, some of which have recently gained attention (Levin et al. 2017, Potter et al. 2021, Satoh 2021). To broaden the current perspective of flight metabolism and to better link the different metabolic pathways it comprises, we have adopted an untargeted metabolomic approach.

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Within the adopted untargeted approach, we focused on the following metabolic pathways: (1) lipid metabolism, (2) amino acid metabolism, and (3) glucose metabolism.

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(1) Lipids are considered the primary energy source during endurance migratory flights (Blem 1976, Stevens 2004), accumulated before the migration journey. Plasma triglycerides (TAGs) are usually elevated during refueling (Jenni-Eiermann and Jenni 1992) but may also increase during flight (Bordel and Haase 1993, Schwilch et al. 1996a). Such TAGs differ in their fatty acid (FA) composition in terms of the length of the carbon chain and the unsaturation levels. Most lipid reserves in migrating birds are polyunsaturated FA (PUFA) and they are usually considered as the preferred fuel for endurance exercise (Maillet and Weber 2006). Two additional metabolites reflecting the physiological state of an individual bird are plasma glycerol, which increases during fasting due to high rates of lipolysis (Jenni-Eiermann and Jenni 1991), and plasma β-Hydroxybutyric acid (BUTY), which increases during fasting owing to ketone formation. The level of BUTY increases shortly after exercise (~20 minutes), indicating post-exercise ketosis that lasts for several hours (Jenni-Eiermann and Jenni 2001). BUTY levels gradually decrease after sufficient rest (~10 hours). Metabolic studies also highlight birds' tolerance to hypoxia, which is indicated by elevated plasma lactate (Faraci 1991), as well as by the post-flight metabolic state, during which birds continue lipolysis at a reduced level to meet the energy demands of resting (Jenni-Eiermann 2017).

(2) The role of protein catabolism in bird migration was thoroughly investigated (Bauchinger and McWilliams 2012). During long-distance flights, birds catabolize not only lipids but also proteins. These proteins originate in the muscles and other internal organs, especially digestive organs (McWilliams and Karasov 2001, Bauchinger and McWilliams 2012). Free amino

acids derived from protein catabolism were previously suggested to serve as substrates for a) gluconeogenesis necessary to meet the brain energy requirements, b) building new energy stores when the fat stores are depleted, and c) maintain water balance during nonstop flights (Gerson and Guglielmo 2011). Additionally, catabolizing protein is known to have antioxidative capacity benefits, as amino acids' bioactive properties are liberated during detaching from the parent protein in which these peptides are usually inactive (Dai et al. 2017).

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(3) An additional metabolite of high importance in birds is glucose. Birds are naturally hyperglycemic, maintaining approximately twice the plasma-glucose concentration of mammals at equivalent size while using mechanisms of insulin resistance (Braun and Sweazea 2008). Although the ultimate causation of this phenomenon is largely unknown, recent studies have suggested that hyperglycemia and insulin resistance are related to the drop of oxygen concentrations in the atmosphere at the Permian–Triassic (PT) boundary, forcing theropods to lose certain genes to maximize their efficiency of oxygen usage (Satoh 2021). Indeed, omentin and insulin-sensitive glucose transporter 4 (GLUT4) are considered missing or unfunctional in the bird genome (Braun and Sweazea 2008, Luo et al. 2023). Because these gene products play essential roles in maintaining insulin sensitivity, this loss probably forced theropods to become insulin resistant (Satoh 2021). These high blood glucose levels were also suggested to be correlated with the high metabolic rate and body temperature of birds associated with the extreme energetic requirements of powered flights (Clarke and Rothery 2008, Clarke and Pörtner 2010). Blood glucose levels usually increase after endurance flight (Viswanathan et al. 1987, Schwilch et al. 1996b, Abdel-Rachied et al. 2014), Yet it is not clear if this hyperglycemia represents an adaptive metabolic mechanism or constraint.

We quantified the plasma metabolome of two most common migratory warbler species in Israel: The Eurasian Blackcap (*Sylvia atricapilla*) and Lesser Whitethroat (*Curruca curruca*). Although related, these two species differ in their breeding areas and habitat selection during a stopover. Differences are also manifested in their diet preferences during migration, as the Eurasian Blackcap is more restricted to water consumption (Sapir et al. 2004). Birds were sampled at two previously studied stopover sites dominated by Pistacia trees, bearing fat-rich fruits during autumn (Domer et al. 2018), namely Midreshet Ben-Gurion (hereafter BGS) and Ein-Rimon (hereafter ER), located in arid and semi-arid areas, respectively. While ER is a planted homogeneous *Pistacia atlantica* grove, BGS is a mixed pistachio grove comprising four primary species: *Pistacia atlantica*, *Pistacia chinensis*, *Pistacia vera*, and *Pistacia lentiscus*. Birds were also sampled at a third site, located in the semi-arid area of Israel, ~11 km south of ER, and mainly populated by autumn-blooming Eucalyptus trees (Negev Brigade Monument, hereafter AN, (31º16'N 34º49'E)). Previous research showed that fuel accumulation and recapture rates were substantially lower in BGS compared with ER (Domer et al. 2018). These findings may suggest that most birds captured at BGS (arid region) are leaving soon after arrival, and are captured several hours after landing, and most of those caught at ER (semi-arid area) are at a resting/refueling state. Therefore, we hypothesized that plasma metabolome varies among sites, depending on the type of the primary food source (fat-rich fruits vs. nectar) and the physiological state of staging birds (either well rested or landed during the previous night).

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Methods

We conducted metabolomic profiling of the Eurasian Blackcap (N=43, Table 1) and the Lesser Whitethroat (N=30, Table 1). Birds were captured for 3 hours during the morning using

mist-nets, opened at first light for three hours. Captured birds were individually tagged with numbered aluminum leg rings, weighed to ±0.1 g with a digital balance, and measured for wing length. Soon after (a few minutes after capture), a blood sample of 0.1ml was extracted from the bird's jugular vein using 25G insulin needle and heparinized tubes. The blood samples were then stored on ice for several hours, before being centrifuged at 10,000 RPM for 10 min at 4°c. Extracted plasma was maintained at -80°c until processing for metabolomic analyses.

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Metabolomic analyses

Lipid and Polar Metabolite Extraction Protocol

Metabolites were extracted from 50 µl of plasma using a protocol described by Hummel et al. (2011). In brief, metabolites from each aliquot were extracted with 1 ml of pre-cooled (−20°C) extraction buffer (homogenous methanol/methyl-*tert*-butyl-ether [1:3] mixture). After 10 min incubation at 4°C and sonication for 10 min in a sonic bath, 500 µl of methanol/water [1:3] mixture was added. Samples were then centrifuged (5 min, 14 000 *g*), leading to a lipophilic and polar phase forming. Five hundred microliters of the lipophilic (upper) phase and 150 µl of the polar phase were collected and dried under a vacuum. The lipophilic phase was resuspended in 200 µl of ACN/isopropanol and used for lipid analysis. The polar phase residue was derivatized for 120 min at 37°C (in 50 µl of 20 mg ml−1 methoxyamine hydrochloride in pyridine) followed by a 30-min treatment at 37°C with 50 µl of MSTFA (with fatty acid methyl esters) and was used for gas chromatography–mass spectrometry (GC–MS) analysis.

Lipid Profiling

Samples were processed using UPLC-FT-MS (Hummel et al. 2011) on a C8 reverse-phase column (100 × 2.1 mm × 1.7 µm particle size, Waters) at 60°C. The mobile phases consisted of 1% 1 M NH_4OAc and 0.1% acetic acid in water (buffer A), and acetonitrile/isopropanol (7:3, UPLC grade BioSolve) supplemented with 1 M NH4Ac and 0.1% acetic acid (buffer B). The following gradient profile was applied: 1 min 45% A, 3 min linear gradient from 45% A to 35% A, 8 min linear gradient from 25% to 11% A, 3 min linear gradient from 11% to 1% A. Finally, after washing the column for 3 min with 1% A the buffer was set back to 45% A and the column was re-equilibrated for 4 min, leading to a total run time of 22 min. The flow rate of the mobile phase was 400 µl/min.

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The mass spectra were acquired using a Q-Exactive mass spectrometer (Thermo Fisher, http://www.thermofisher.com) equipped with an ESI interface. All the spectra were recorded using altering full-scan mode, covering a mass range from 150–1500 *m*/*z* at a capillary voltage of 3.0 kV, with a sheath gas flow value of 60 and an auxiliary gas flow of 35. The resolution was set to 30000 with 3 scans per second, restricting the Orbitrap loading time to a maximum of 100 ms with a target value of 1E6 ions. The capillary temperature was set to 150°C, while the drying gas in the heated electrospray source was set to 350°C. The skimmer voltage was held at 25 V while the tube lens was set to a value of 130 V. The spectra were recorded from minute 1 to minute 20 of the UPLC gradients.

Processing of chromatograms, peak detection, and integration was performed using REFINER MS 14.0 (GeneData, http://www.genedata.com) or Xcalibur (Version 3.1, Thermo Fisher, Bremen, Germany). In the first approach, the molecular masses, retention time, and associated peak intensities of the sample were extracted from the raw files, which contained the

full-scan MS. Processing MS data included removing the fragmentation information, isotopic peaks, and chemical noise. Further peak filtering on the manually extracted spectra or the aligned data matrices was performed. Obtained features (*m*/*z* at a certain retention time) were queried against an in-house lipid database (Lapidot-Cohen et al. 2020).

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Polar Metabolite Analysis

The GC–MS system was a gas chromatograph coupled to a time-of-flight mass spectrometer (Pegasus III, Leco). An autosampler system (PAL) injected the samples. Helium was used as carrier gas at a constant flow rate of 2 ml s−1 , and gas chromatography was done on a 30-m DB-35 column. The injection temperature was 230°C, and the transfer line and ion source were set to 250°C. The initial temperature of the oven (85°C) increased at a rate of 15°C min−1 up to a final temperature of 360°C. After a solvent delay of 180 s, mass spectra were recorded at 20 scans s−1 with *m*/*z* 70–600 scanning range. Chromatograms and mass spectra were evaluated by using Chroma TOF 1.0 (Leco) (Schauer et al. 2008) together with TargetSearch (Cuadros-Inostroza et al. 2009) and Xcalibur Software (Thermo Scientific). Data for the lipid and polar metabolites is available at Dryad (Domer Adi 2023).

Statistical analyses

To test for differences in the plasma metabolite composition between birds sampled at the different stopover sites, we used non-metric multidimensional scaling (nMDS) ordinations of the Bray-Curtis dissimilarity matrix, followed by PERMANOVA and SIMPER analyses. The latter allowed quantifying the contribution of different metabolites to the observed inter-site variation. To test for differences in plasma BUTY and TAG levels, we used a generalized linear model (glm)

with normal distribution for each response variable, using the site as a categorical variable, and body condition (derived from the residuals of regressing individuals' body mass against wing length) as a covariate. To test for differences in the levels of specific metabolites (annotated amino acids and non-annotated metabolites detected by SIMPER analyses) among sites, we used multivariate analysis of variance (MANOVA), with annotated metabolites as response variables, the site as a categorical variable, and body condition (derived from the residuals of regressing individuals' body mass against wing length) as a covariate. All statistical analyses were performed in R 3.4.4 (Team 2013).

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Results

Polar metabolites

Bird plasma samples were analyzed using GC-MS, generating 414 distinct metabolites. To test for inter-site differences in the composition of these metabolites, we used a non-metric multidimensional scaling (nMDS) ordination of the Bray-Curtis dissimilarity matrix, followed by a PERMANOVA and SIMPER analysis. In both warbler species, the composition of blood polar metabolites varied significantly among sites (Fig. 1; PERMANOVAs: $F_{2,37}$=36.135, P<0.001, R^2=0.629 and $F_{2,27}$=15.653, P<0.001, R^2=0.504 for Eurasian Blackcap and Lesser Whitethroat, respectively). The polar metabolic profile of Eurasian Blackcap varied significantly with body condition, derived from the residuals of regressing body mass against wing length ($F_{1,37}$=3.872, P=0.038, R^2=0.034) but not that of Lesser Whitethroat ($F_{1,27}$=0.563, P=0.567, R^2=0.009). In both species, the interaction between site and body condition was not significant ($F_{2,37}$= 0.814, P=0.496, R^2=0.014 and $F_{2,27}$=1.617, P=0.179, R^2=0.052 for Eurasian Blackcap and Lesser Whitethroat, respectively). Pairwise comparisons revealed that the polar metabolic profile, characterizing birds

in BGS varied significantly from that of birds in ER (P=0.003 and P=0.003 for Eurasian Blackcap and Lesser Whitethroat, respectively) and AN (P=0.003 and P=0.003 for Eurasian Blackcap and Lesser Whitethroat, respectively), but not between ER and AN (P=0.078 and P=0.063 for Eurasian Blackcap and Lesser Whitethroat, respectively), although both might be considered as marginally non-significant.

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SIMPER analysis identified ten metabolites contributing most to the dissimilarity among sites. These metabolites were identical in both warbler species and appeared significantly different at all inter-site pairwise comparisons. In Eurasian Blackcap, these metabolites contributed 48.5% (ER vs. BGS), 46.2% (AN vs. BGS), and 51.6% (AN vs. ER) to the inter-site dissimilarity. In Lesser Whitethroat, these metabolites contributed 49.6% (ER vs. BGS), 48.1% (AN vs. BGS), and 49.9% (AN vs. ER) to the inter-site dissimilarity. Among these ten metabolites, we annotated six metabolites: lactic acid, malic acid, glycerol, glycerol 3-phosphate, glucose and alanine (Fig. 2). The level of these metabolites varied significantly among sites (approx. $F_{12,66}$=4.615, P<0.001, and approx. $F_{12,46}$=6.575, P<0.001, for Eurasian Blackcap and Lesser Whitethroat, respectively; Tables 1S and 2S, Supplementary material). The intensity of these metabolites did not vary significantly as a function of body condition (approx. $F_{6,32}$=0.734, P=0.626, and $F_{6,22}$=1.897 P=0.127, for Eurasian Blackcap and Lesser Whitethroat, respectively), Nevertheless, the interaction between site and body condition was significant for Lesser Whitethroat (approx. $F_{12,46}$=2.253 P=0.024) but not for Eurasian Blackcap (approx. $F_{12,66}$=0.944, P=0.510). In Eurasian Blackcap, the levels of all six metabolites were significantly higher in BGS than in ER and AN (Tukey HSD p<0.01), while in Lesser Whitethroat, the level of lactic acid, alanine and glycerol 3-phosphate, were significantly higher in BGS than in ER and AN. A similar pattern was evident for malic acid with significant differences only between BGS and AN (P=0.006), glycerol with all pairwise comparisons being

significant (P<0.05), and glucose with significant differences only between BGS and ER (P=0.003).

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Amino acids

Multivariate analysis of variance (MANOVA) followed by univariate tests, indicated that the intensities of all 12 annotated plasma amino acids were significantly higher in BGS, compared with the other two sites (approx. $F_{26,52}$=3.291, P<0.001 and approx. $F_{26,30}$=2.486, P=0.008, for Blackcap and Lesser Whitethroat, respectively; tables 3S and 4S, supplementary material). Additionally, individuals' body condition did not significantly affect the plasma amino acids of both, Eurasian Blackcaps (approx. $F_{13,25}$=2.077, P=0.057) and Lesser Whitethroats ($F_{13,14}$=0.708, P=0.729), though the trend for Blackcaps is only marginally insignificant. Lastly, the interaction between site and body condition was not significant for both species (approx. $F_{26,52}$=0.940, P=0.557 and approx. $F_{26,30}$=1.379, P=0.197, for Blackcap and Lesser Whitethroat, respectively)

Lipophilic profile

An nMDS ordination of the Bray-Curtis dissimilarity matrix, followed by a PERMANOVA indicated that in both warbler species the composition of lipophilic metabolites varied significantly among sites (Fig. 1S, supplementary material; PERMANOVAs: $F_{2,37}$=6.6465, P>0.001, R^2=0.237 in Eurasian Blackcap, and $F_{2,27}$=3.540, P=0.001, R^2=0.183 in Lesser Whitethroat). Additionally, individuals' body condition, derived from the residuals of regressing body mass against wing length, significantly affected the lipophilic profile of both species ($F_{1,37}$=2.523, P=0.038, R^2=0.041 in Eurasian Blackcap, and $F_{1,27}$=2.951, P=0.017, R^2=0.076 in Lesser Whitethroat). The interaction between site and body condition was not significant in both

261 species ($F_{2,37}$=1.601, P=0.114, R^2=0.057 in Eurasian Blackcap, and $F_{2,27}$=0.832, P=582, R^2=0.043
262 in Lesser Whitethroat). Pairwise comparisons revealed that the lipophilic profile of Blackcaps was
263 significantly different when staging at ER compared with AN and BGS (P=0.009, and P=0.003 for
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264 Eurasian Blackcap and Lesser Whitethroat, respectively). Similarly, for Lesser whitethroats,
265 pairwise comparison revealed significantly different lipophilic profile when staging at AN
266 compared with ER and BGS (P=0.003 and P=0.036 for Eurasian Blackcap and Lesser Whitethroat,
267 respectively).

268 SIMPER analysis identified ten metabolites contributing most to the dissimilarity among
269 sites. In both species, and at all inter-site pairwise comparisons This list of lipids was comprised
270 of 6-7 TAGs (50-54 carbons, with varying saturation levels of 1-5 double bonds) and 2-4
271 phosphatidylcholine (34-38 carbons, with varying saturation levels of 1-4 double bonds). In
272 Eurasian Blackcap, these lipids contributed 31.8% (ER vs. BGS), 32.3% (AN vs. BGS), and 31.7%
273 (AN vs. ER) to the inter-site dissimilarity. In Lesser Whitethroat, these metabolites contributed
274 33.6% (ER vs. BGS), 31.4% (AN vs. BGS), and 36.2% (AN vs. ER) to the inter-site dissimilarity.
275 The annotated lipids mean intensities varied, and consistent pattern across sites could not be
276 detected. We therefore added additional analyses of TAGs and BUTY.

277 To further examine the plasma lipids, we quantified the accumulated level of plasma TAGs
278 (Fig. 3), manifested as intensities. Total TAG intensities were not significantly different among
279 sites, for both species ($F_{2,37}$=0.995, P=0.379 and $F_{2.26}$=0.689, P=0.511, for Eurasian Blackcap and
280 Lesser Whitethroat, respectively). Body condition did not significantly affect the total TAG
281 intensities for both species ($F_{1,37}$=2.050, P=0.161 and $F_{1,26}$=0.044, P=0.836, for Eurasian Blackcap
282 and Lesser Whitethroat, respectively). We further compared PUFA TAGs (with 6-8 double bonds

within the TAG) to explore potential differences not exposed by total TAG comparison. The patterns of PUFA TAGs were consistent between the two warbler species: PUFA TAG varied among sites ($F_{2,37}$=6.122, P=0.005 and $F_{2,26}$=3.163, P=0.059, for Eurasian Blackcap and Lesser Whitethroat, respectively), though the trend for Lesser whitethroat could be considered as marginally insignificant. However, there was no effect of body condition on the TAGs intensity ($F_{1,37}$=0.052, P=0.820 and $F_{1,26}$=0.208, P=0.652, for Eurasian Blackcap and Lesser Whitethroat, respectively). Specifically, PUFA TAG intensities were higher in ER than in AN (t_{37}=2.16, P=0.031, and t_{26}=2.33, P=0.020 for Eurasian Blackcap and Lesser Whitethroat, respectively) and BGS, with the latter being marginally non-significant in Lesser Whitethroat (t_{37}=2.16, P=0.031, and t_{26}=1.7, P=0.088 for Eurasian Blackcap and Lesser Whitethroat, respectively). No significant differences in PUFA TAG intensity were detected between BGS and AN (t_{37}=0.094, P=0.926, and t_{26}=0.655, P=0.518 for Eurasian Blackcap and Lesser Whitethroat, respectively).

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β-Hydroxybutyric acid

We focused on an additional metabolite, β-Hydroxybutyric acid (BUTY), which was not included in the list of metabolites detected by SIMPER but is considered to play a key role in avian metabolism (Jenni-Eiermann and Jenni 1991, Guglielmo et al. 2005), particularly during fat accumulation and ketogenesis. In both species, BUTY levels varied among sites ($F_{2,37}$=3.480, P=0.041, and $F_{2,26}$ = 8.304, P=0.002, for Eurasian Blackcap and Lesser Whitethroat, respectively; Fig. 4). Moreover, BUTY levels were significantly lower in AN compared with ER and BGS for both the Blackcaps (t_{37} = 2.330, P=0.025 and t_{37}=2.911, P=0.006, for comparing AN with ER and BGS, respectively) and the Lesser Whitethroats (t_{26} = 2.508, P=0.019 and t_{26}=3.630, P=0.001, for comparing AN with ER and BGS, respectively). Body condition significantly affected the plasma

BUTY levels of the Blackcaps ($F_{1,37}$=4.580, P=0.039) with the interaction between body condition and site also being significant ($F_{2,37}$ = 5.044, P=0.039), however, body condition did not affect plasma BUTY levels of the lesser whitethroats ($F_{2,37}$ = 0.067, P=0.798).

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Discussion

We conducted a comparative field study to quantify the plasma metabolome of two common migratory passerine species at three different stopover sites in the northern Negev desert of Israel during autumn migration. We found that both warbler species' polar and lipophilic metabolites varied significantly among sites. The inter-site variation in the polar metabolites can be mainly attributed to the inter-site variation in the birds' physiological state. That is, the above-mentioned metabolites, differentiating among sites are mainly related to fasting and flight recovery. In this way, lactic acid, glucose, and glycerol are examples of metabolites that were previously demonstrated to vary between birds before and after resting (Viswanathan et al. 1987, Jenni-Eiermann and Jenni 1992). Our previous research efforts (Domer et al. 2018, 2021) have shown that during autumn migration, both recapture and fuel accumulation rates are higher in ER than in BGS. These findings, in combination with the results presented here, strongly suggest that most birds at BGS leave soon after arrival (i.e., do not spend another night at this site) while most birds in ER are at a resting/refueling state. Importantly, we could not detect significant correlation of body condition with the annotated metabolites identified by SIMPER, as well as with the amino acids, except for two distinct cases, glycerol and isoleucine, both were significantly different across body condition only for Blackcaps, with the latter also showing a significant site by body condition interaction. The inter-site variation in the lipophilic profiles of birds was harder to interpret and is suspected to reflect the variation in the primary food source.

The body condition of the birds seemed to significantly affect the plasma metabolic profile or the levels of metabolites only in distinct occasions and not for both species. That is, while body condition may have affected the plasma level of some metabolites, the variation in plasma polar metabolites is mainly associated with differences in site characteristics. Importantly, all birds captured at the arid site (BGS) were in a physiological state indicating only a short rest after flight (e.g., high lactic acid) and are suspected to have landed during the previous night. Lastly, the inter-site variation in the polar metabolic profile was mainly generated by ten metabolites, six of which were successfully annotated. Below, we discuss the involvement of these six metabolites in critical metabolic pathways activated during stopovers.

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Stopover metabolism

The polar metabolites found to vary among stopover sites were identical in both species. These metabolites mainly participate in four energy metabolism pathways: (1) fatty acid oxidation, (2) protein catabolism, (3) glucose-alanine (Cahill) cycle, and (4) lactic acid (Cori) cycle. These pathways, activated during fasting and endurance exercise, often operate simultaneously during migration. The primary energy source for long flights is derived from subcutaneous lipids. TAG degradation in the cytosol produces glycerol and glycerol 3-phosphate, which can also be a precursor for gluconeogenesis (Robergs and Griffin 1998). Fat stores are essential for birds (Pond 1978, Guglielmo 2010), as they do not carry large glycogen stores, probably due to the high cost of maintaining such hygroscopic storage molecule (Hickey et al. 2012).

In addition to glycerol, another energy source can be amino acids, derived from protein catabolism. Such catabolism occurs during flight and starvation in flight muscles, but also in the liver and other digestive organs (Bauchinger and McWilliams 2010). We found that the intensities

of plasma amino acids are higher in birds that landed at BGS, which are suspected to have landed during the previous night, compared to the other sites accompanied by elevated plasma glucose. Previous research has thoroughly discussed the role protein catabolism plays during migration flight and fasting (Bauchinger and McWilliams 2012). Given that all suggested hypotheses are not mutually exclusive, and in the light of the high intensities of plasma amino acids in birds from BGS, which are likely to have landed a few hours prior capture, we suggest that an additional main pathway for these amino acids is to serve as precursors for cycling glucose in the liver via gluconeogenesis (Fig. 5).

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During fasting, peripheral organs become more catabolic, and such protein catabolism can support stress and healing processes by cycling glucose towards Cahill and Cori cycles (Deutz et al. 1992, Soeters and Soeters 2012). Furthermore, the cycled glucose can also facilitate reducing equivalent NADPH, which is necessary to maintain redox potential (Levin et al. 2017), a common result of endurance exercise. We, therefore, suggest that protein degradation facilitates the metabolic cycling of glucose to support physiological stress.

Cahill (alanine) and Cori (lactic acid) cycles are responsible for cycling nutrients between the skeletal muscles and liver. In the Cori cycle, the lactate, produced by anaerobic glycolysis in muscles, is transported to the liver and converted to glucose, then returns to the muscles and metabolized back to lactate, preventing the accumulation of blood lactate. The contribution of lactate to overall glucose production increases with fasting duration (Katz and Tayek 1998). Nonetheless, fasting requires utilizing substrates already present in the body. For birds, subcutaneous lipids can provide most energy for long-distance migration (Pond 1978), yet this metabolic pathway occurs alongside protein catabolism (McWilliams and Karasov 2005). In the

Cahill (or alanine) cycle, the nitrogen, generated from amon acid degradation is trans-aminated to pyruvate, forming alanine (Felig 1973), and mobilized to the liver for nitrogen disposal via the urea or uric acid cycle in birds (Milroy 1993). In contrast to the Cori cycle, this pathway causes NAD^+ deficiency, which in turn can be counteracted via the malate shuttle (Mettler and Beevers 1980) or the glycerol-3-phosphate shuttle (Shen et al. 2006). Both are mechanisms for generating NAD^+, and are supported by our data, namely, the higher plasma malic acid, glycerol, and glycerol 3-phosphate, detected in non-rested birds.

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Insulin resistance and hyperglycemia are one of the most important mechanisms for coping with prolonged fasting in animals (Soeters and Soeters 2012). Remarkably, the Cori and Cahill cycles were previously related to insulin resistance (Katz and Tayek 1998, Sarabhai and Roden 2019). Soeters et al. (2021) suggested that cycling glucose metabolites, alongside insulin resistance, are metabolically connected, serving as a beneficial survival response. They also suggested that this pattern leads to fatty acid oxidation and may be a consequence rather than a cause of insulin resistance. Adaptive insulin resistance was previously documented in some animal species, as an adaptation for living in nutrient-limited environments (Houser et al. 2013, Riddle et al. 2018). As flying vertebrates, characterized by extremely high metabolic rates, migrating birds should constantly deal with endurance exercise, even during simple movements, as well as prolonged fasting associated with migration. We suggest that avian insulin resistance and hyperglycemia are mechanisms for recovering from long-endurance flights, despite incapability of feeding.

Ecological perspective

Here we show that the same ten polar metabolites, which largely generate the inter-site dissimilarly in the metabolome of both warbler species, are highly related to the physiological status of the birds, suggesting they have recently landed from long flight. While the detected inter-site variation in the polar metabolic profile could be attributed to variation in the birds' physiological state, this intrinsic factor could not explain the observed inter-site variation in the lipophilic profiles. Most birds in ER and AN were in a resting/refueling state, although these sites offered them different food types (fat-rich fruits and nectar, respectively). Nevertheless, there were significant differences not only in their lipophilic profile but also in their PUFA TAGs, and BUTY intensities, which were higher in ER and are known to increase not only when fasting, but also when feeding on a lipid food source (Smith et al. 2007). We therefore suggest that the lipophilic profile variation between ER and AN should be attributed to the primary food source these two sites provide. Nectar is comprised mainly of sugars dissolved in water which are absorbed quickly into the digestive tract of birds (Tracy et al. 2010). Therefore, the blood glucose associated with nectar consumption may have little or no immediate effect on the respective lipophilic profile compared to the consumption of fat-rich fruits. (Jenni-Eiermann and Jenni 1991)

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Conclusions

Although lipid metabolism is considered as the primary metabolic pathway during long-endurance flights (Ramenofsky 1990, Jenni-Eiermann 2017), the results of blood lipid profiles were hard to interpret, as they contained many lipophilic compounds that do not necessarily relate to lipid metabolism during exercise. Additionally, TAG and BUTY levels were not a good indicator of site quality. These findings are consistent with previous research suggesting that the context of these metabolites may be species-specific or related to food sources (Jenni-Eiermann

and Jenni 1992, Guglielmo et al. 2005, Smith et al. 2007). Essentially, the pathways proposed here to be activated during a stopover indicate a need for flight recovery and suggest that glucose cycling, derived from protein catabolism, plays a vital role in this recovery process. Moreover, this viewpoint also suggests that avian insulin resistance and hyperglycemia have evolved due to endurance exercise, prolonged fasting, and fatty acid oxidation, similar to trauma recovery in other animals.

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Tables

Table 1. Number of Blackcaps and Lesser Whitethroats sampled at each study site.

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Site	Lesser Whitethroat	Blackcap
AN	8	5
ER	11	19
BGS	11	19

Figures

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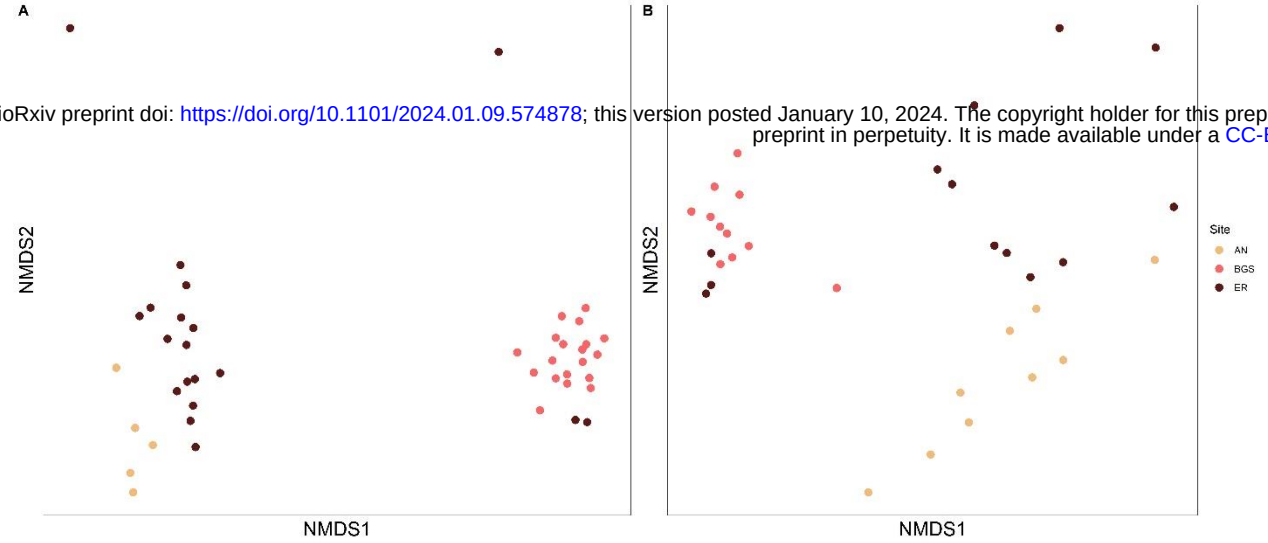

Figure 1: Nonmetric multidimensional scaling ordinations for the Bray-Curtis dissimilarity matrix, demonstrating clear separation in the composition of polar metabolic profile. Eurasian Blackcap (A) and Lesser Whitethroat (B) in the three different stopover sites. Freshly landed vs. rested Eurasian Blackcap (C) and Lesser Whitethroat (D).

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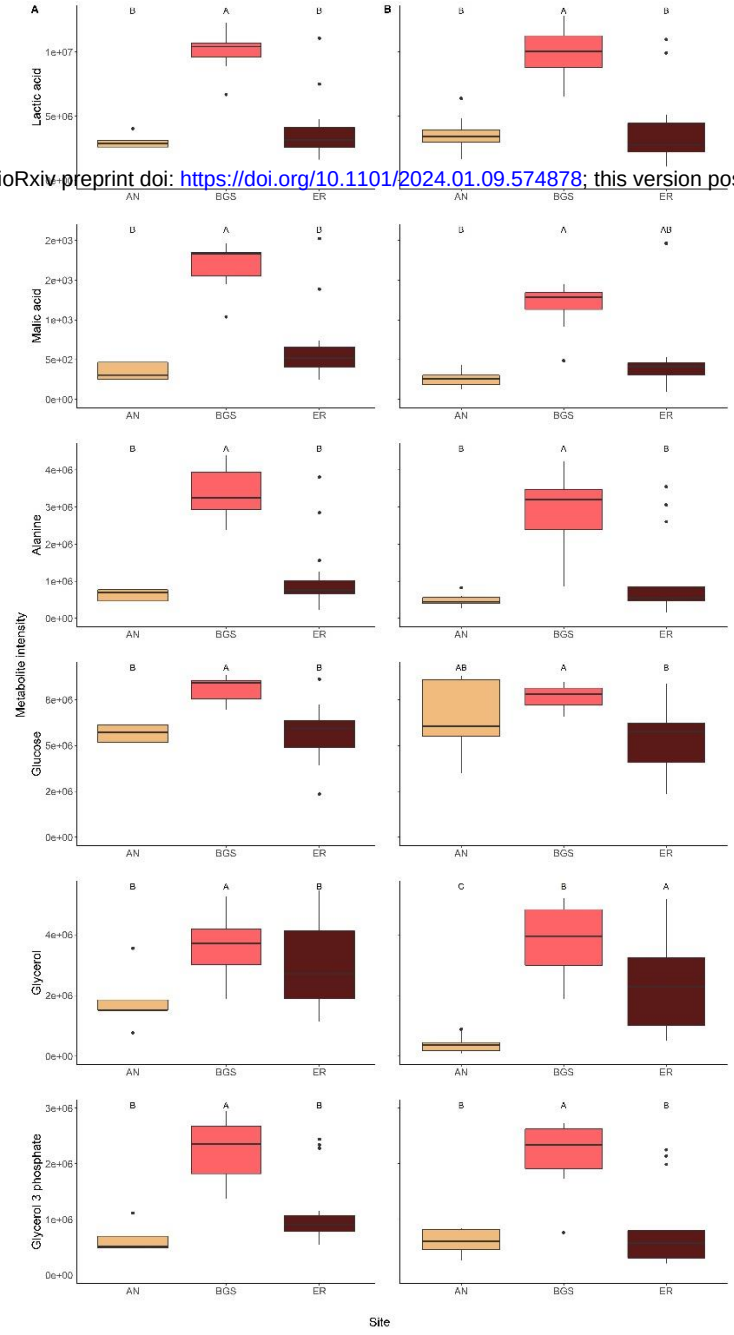

Figure 2: Differences in key polar metabolites in Eurasian Blackcap (A) and Lesser Whitethroat (B) among the three different stopover sites, as detected in SIMPER analyses. Different letters account for significant differences. Within boxes, horizontal lines indicate the median; black dots

show the mean; box boundaries indicate the interquartile range; whiskers indicate minimum and maximum.

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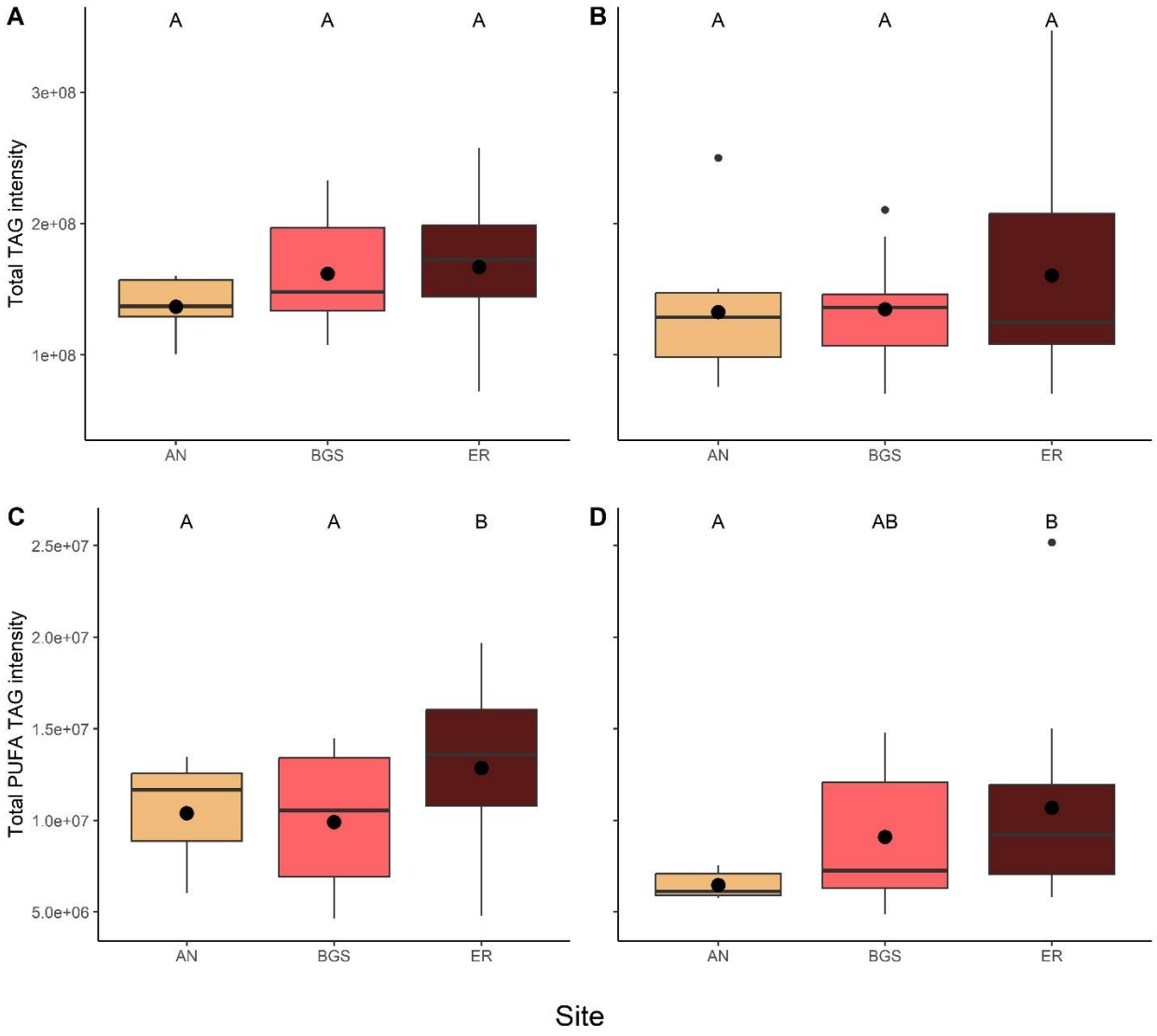

Figure 3: Differences in relative intensity of total TAG in Eurasian Blackcap (A) and Lesser Whitethroat (B), and total PUFA TAG in Eurasian Blackcap (C) and Lesser Whitethroat (D) among the three different stopover sites. Different letters account for significant differences.

441 Within boxes, horizontal lines indicate the median; black dots show the mean; box boundaries
442 indicate the interquartile range; whiskers indicate minimum and maximum.

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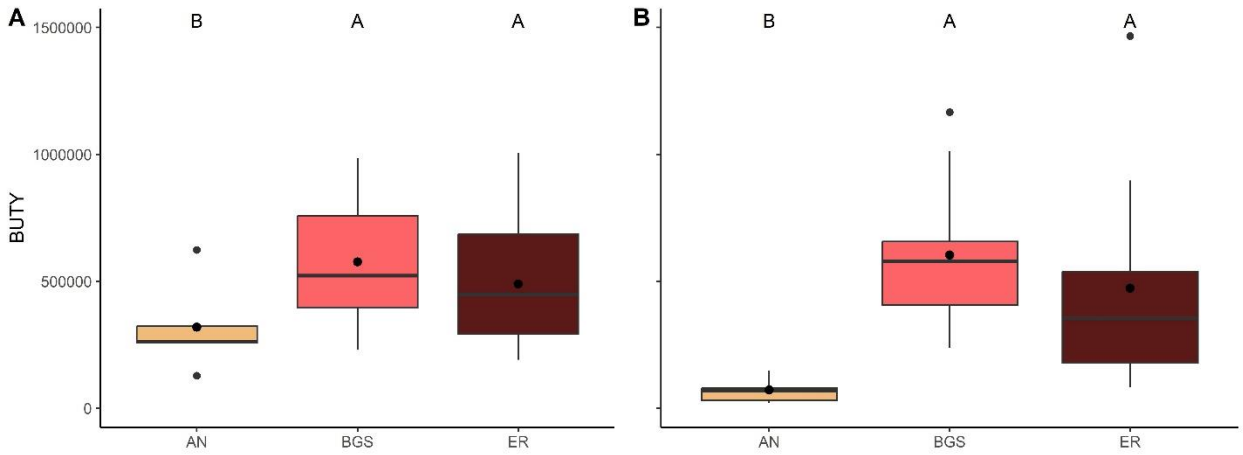

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445 Figure 4: Differences in relative intensity of β-Hydroxybutyric acid (BUTY) in Eurasian Blackcap
446 (A) and Lesser Whitethroat (B) among the three different stopover sites. Different letters account
447 for significant differences. Within boxes, horizontal lines indicate the median; black dots show the
448 mean; box boundaries indicate the interquartile range; whiskers indicate minimum and maximum.

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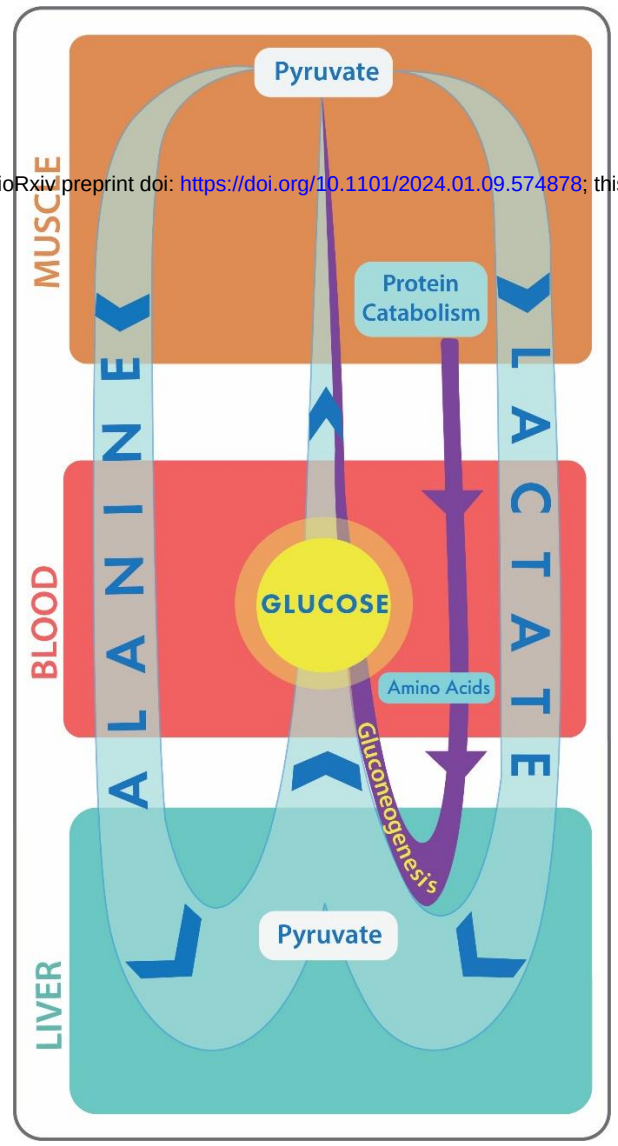

Figure 5: The suggested fate of protein catabolism and elevated plasma glucose during and post long-endurance flights. Free amino acids are delivered to the liver through the bloodstream. These amino acids are then used to produce glucose using gluconeogenesis. Lactic acid is maintained as a result of anaerobic conditions. The alanine cycle is maintained for disposal of the ammonium group through the uric acid cycle. The lack of NAD+ is compensated via the malate and glycerol shuttles. High plasma glucose can also facilitate repair mechanisms for high oxidative stress.

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All authors declare to have no competing interests.

The data that support the findings of this study will be made openly available in a repository.