Night Migratory Songbirds Exhibit Metabolic Ability to Support High Aerobic Capacity during Migration

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ABSTRACT: Aerobic metabolism in night migratory songbirds exhibit seasonal plasticity, which depends not only on annual life history stages (LHSs), viz., migratory/nonmigratory or breeding/nonbreeding, but also on the time of the day. Initially, we studied daily changes in behavior/physiology alongside aerobic metabolism intermediates using gas chromatography–mass spectrometry-based chemometric analyses of serum of migratory male redheaded buntings during low-energy wintering, that is, the nonmigrating LHS. Then, the metabolic phenotype of nonmigrating birds was compared with that of photostimulated migrating buntings, the latter representing the high-energy LHS. Diurnal changes such as daytime feeding and activity were reflected by increased fatty acid (FA, viz., palmitic, oleic, and linoleic acids) levels and protein catabolites, whereas higher night-time levels of short-chain FAs indicated lipolysis in night-fasted birds. High night-time levels of taurine, a sulfur amino acid, suggested the endogenous metabolite rendering an adaptive advantage to hyperglycaemic night activity during migration. While it is day-active during migration, night activity during migration is expected to alter the liver's protective ability to combat metabolic stress through high aerobic capacity during migration. This study elucidates putative “serum biomarkers” with a protective role in stress accrued by enhanced aerobic capacity requirements at the organismal level.

INTRODUCTION

Birds fly to survive. Nearly one-fifth of avifauna undertakes perilous fortitude of migration (arctic tern, up to 90,000 km every year), exhibiting an epitome of aerobic exercise efficiency. A combination of factors such as annual changes in day length, decreasing temperatures, lowered food supplies, or genetic predisposition can trigger migration1 in small passerines blackpoll warbler,2 Setophaga striata, and northern wheatear,3 Oenanthe oenanthe, enabling them to undertake multiday flights relying on stored energy.

Most studies on migration mystery stick to the “fat to fuel” theme.4,5 To maximize need-based fat utilization efficiency, organ-specific cellular differentiation mediates fat accumulation before migration so that the adipose tissue accumulates fat in different locations and multi-substages.6 Migratory birds exhibit species differences in fat mobilization from adipose tissue because of seasonal difference capability of the liver to metabolize fat. This seasonal flexibility in birds’ ability to mobilize fat from adipose tissue to the liver and muscles varies among species. Also, migratory birds can redefine aerobic metabolism depending on the migratory or nonmigratory state.7 Figure 1a shows simplified annual life history stages (LHSs) of the night migratory songbird redheaded bunting (Emberiza bruniceps) which overwinters in India and returns (spring migration) to temperate breeding grounds for breeding.8–10

A day-active nonmigrating bunting changes to predominant night activity during migration. While it is day-active during the nonmigratory stage, it eats in the day. Food consumed in the day replenishes fuel stores used during the night. Therefore, it is expected that the liver exports nutrients during night fasting. However, this condition is expected to alter during the energy-intense state of migration, when there is continued yet selective mobilization of lipids by the liver.

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is the most important organ for lipid synthesis. An increase in consequences. Equivocally, in birds and mammals, the liver condition and have the ability to overcome fat-burn exhibit increased aerobic capacity under an overweight migratory birds are, therefore, particularly important as birds after accumulating fat, that is, becoming overweight. Studies on annual LHS-speci

These behavioral and physiological transitions between the LHS result from dynamic regulation of cell-autonomous circadian clocks and key biochemical pathways. Efficient energy acquisition from body fat needs differential regulation of metabolic pathways which has adaptive dependency on seasonal energy demands. We hypothesized that seasonal plasticity in diurnal metabolic pathways of night migratory songbirds is associated with time—energy budget activity that is annual LHS-specific.

How the birds achieve plasticity in their seasonal physiology is even more interesting from a view that diurnal birds preparing for migration overeat to accumulate fat depots. The fat is differentially deposited across the body so that the bird acquires double of its original weight, following which it undertakes migratory journey. Then, the bird loses weight during migration “exercise”, reaching the original “thin” body by the end of the migratory LHS. In contrast, in mammalian model species, high aerobic capacity and hence endurance are positively related to the lean body phenotype. Birds migrate after accumulating fat, that is, becoming overweight. Studies on migratory birds are, therefore, particularly important as birds exhibit increased aerobic capacity under an overweight condition and have the ability to overcome fat-burn consequences. Equivocally, in birds and mammals, the liver is the most important organ for lipid synthesis. An increase in the liver’s ability to metabolize fat into “smaller molecules” requires a substantial increase in mitochondrial mass and timed upregulation of fat oxidation pathway enzymes, that is, aerobic metabolism. In birds, fatty acid (FA) uptake in the liver is followed by metabolite transportation to the muscles.

In the past decade, molecular docking studies have identified FA binding proteins (FABPs) as intracellular transporters for the endocannabinoids, implicated in relief from stress, pain, and inflammation. Flight training is important in migrating western sandpiper (Calidris mauri) to stimulate FABP expression. FABP coexpresses with citrate synthase, a measure for aerobic capacity of birds. Migrating redheaded buntings also express higher mRNA levels of FABP during spring migration.

The present study was aimed to investigate diurnal variation of metabolic dependence, that is, day—night changes in selective metabolite requirements were studied in night migratory songbirds E. bruneiceps. Diurnal metabolic changes under the simulated nonmigrating LHS and comparison of seasonal biochemical dynamics of songbirds during spring migratory with the nonmigrating LHS were attempted. Many bird migration studies involving metabolomic assays are reported, for example, study on red knot (Calidris canutus) included assay of three metabolites, viz., triglycerides, uric acid, and β-hydroxy-butyrate. Birds’ energy budgeting has parametric influence over power and endurance of flight. Classic biochemical tools such as assay of metabolites involved in food assimilation and utilization pathways and alteration in the flux along key steps of energy metabolism can reliably explain transitions in organismal aerobic capacity. Metabolite fluctuations also portray outputs from a thorough flux of information from multiple regulatory level inputs such as differential gene expression and post transcriptional/post translational modifications during different LHSs in a seasonal species.

■ RESULTS

Daytime activity and lower metabolic rate during the night reflect in metabolic adjustment of nonmigrating buntings. Buntings exhibited daily rhythm in activity, food intake, and surface body temperature (SBT) (Figure 2a).

Under simulated nonmigrating conditions, daily perch hopping started and ended with the lights on and lights off in day-active buntings (t = 15.52 and p < 0.0001). Similarly, body temperature decreased (Figure 2a) in the night (F(5,47) = 4.43, p = 0.025, and one-way ANOVA followed by the Newman–Keuls multiple post hoc test).

Circulating metabolites that discriminated bunting’s metabolic diurnality are presented in Figure 2a. These included significantly higher levels of mono- and poly-unsaturated FAs (Figure 2b—d, MUFA/PUFA), viz., palmitic acid (palmitate, 16:0; p = 0.0011), oleic acid (oleate) (18:1; p = 0.0325), and linoleic acid (linoleate, 18:2, p = 0.0022; Mann Whitney test) in the daytime. Proteinogenic amino acid proline (Figure 2e) was significantly (p = 0.0011) higher during the day.

Higher night abundance of two TCA intermediates was observed. Oxalic acid exhibited the trend for (p = 0.4429, Figure 2f) higher night abundance but was statistically insignificant. Isocitric acid (p < 0.05, Figure 2g) levels were significantly higher in the night. The reduced isocitrate dehydrogenase and citrate synthase activity in night-fasted birds could be due to the reduced metabolic rate. Short-chain FA, butanoic acid (p = 0.0022, Figure 2h), concentration was higher in the night (t = 4.05, p < 0.005). Serum metabolites of individual buntings showed diurnal variations within the group (Figure 2j).
Conserved immune-protective metabolites are elevated to meet high energy demand during migratory metabolic exercise in buntings.

Nonmigrating buntings were day-active, while migrating birds exhibited behavioral shift (\(F_{23,480} = 3.554, p < 0.0001\), and two-way repeated measure, RM-ANOVA, Figure 3b) by becoming predominantly night-active (resembling Zugunruhe in wild conspecifics, also see Jain and Kumar, 1995) states (summary Figure 3a). Two-way RM-ANOVA revealed significant variation in the daily food consumption pattern (Figure 3c) with respect to the LHS (\(F_{9,60} = 56.88\) and \(p < 0.001\)) and time of the day (\(F_{1,60} = 12.51\) and \(p < 0.0001\)) and interaction between the LHS and time (\(F_{9,60} = 19.79\) and \(p < 0.0001\)). SBT of migrating buntings was higher (\(F_{1,70} = 37.18\) and \(p < 0.001\), Figure 3d) than that of nonmigrating buntings. Levels of metabolites not only differed among nonmigrating and migrating LHSs but also exhibited differences in the time of day/night: “lights on” or the beginning of the day, ZTO (ZT = Zeitgeber time i.e., time of the external cue), ZT4–4 h from lights on, and so on up to ZT 24/0. Metabolites of carbohydrate catabolism, viz., glycolysis, that is, pyruvate (Figure 3b), in migrating buntings were higher few hours before night and midnight (ZT12–10 = 3.38, \(p = 0.007004\); ZT16–10 = 3.21, \(p < 0.009333\); and ZT0–10 = 4.991, \(p < 0.000545\)), and acetate (Figure 3c) levels were higher during the night (ZT0–10 = 3.287, \(p < 0.008185\); and ZT20–10 = 2.819, \(p < 0.048\)). Also, daytime (ZT8) pyruvate levels are higher (\(t = 2.05, p < 0.05\), and paired \(t\)-test) than night (ZT16) levels. Mean daily levels of acetic acid (\(t = 2.54, p < 0.05\), and paired \(t\)-test), but not pyruvic acid (\(t = 1.77, p = 0.06\), and paired \(t\)-test), were significantly higher in migrating than nonmigrating buntings. Food-derived FA hexanoate (Figure 3d, caproic acid, a saturated medium-chain FA) and liver-synthesized butanoate (Figure 3e, SCFA) which yield acetoacetate to enter \(\beta\)-oxidation of FAs were significantly higher (hexanoate-ZT16–10 = 3.284, \(p < 0.01\); ZT20–10 = 5.646, \(p < 0.001\); butanoate-ZT8–10 = 4.23, \(p < 0.001\); ZT12–10 = 2.832, \(p < 0.01\); ZT16–10 = 2.473, \(p < 0.05\); and ZT20–10 = 3.559, \(p < 0.001\)) throughout the day. Palmitoylethanalamide (PEA) (Figure 3f), a glycerophospholipid biosynthesis intermediate carboximidic acid, was high during the midday (\(t = 4.489\) and \(p < 0.005\)) and midnight (\(t = 2.913\) and \(p < 0.05\)) during the migratory LHS.

Pyridoxal phosphate (PLP) was significantly (\(t = 5.589\) and \(p < 0.0001\)) higher throughout 24 h during the migratory LHS (Figure 3g). PLP also mediates the formation of free cysteine from cystathionine, which in turn is a metabolite formed by reaction of homocysteine and serine.

Figure 2. Daily changes (a–j) in behavior, physiology, and metabolism of nonmigratory (wintering) redheaded bunting E. bruniceps. (a) Summary diagram showing 24 h day with light (open arc)/dark (closed arc), active (open wing)/resting (closed wing) birds, higher/lower (cyan blue/gray thermometers) body temperatures, feeding (open beak)/nonfeeding (closed beak) phases, and daily phases (black curves) of elevated concentration of different metabolites during daytime/night time. (b–i) Concentrations (mean + SE) of circulating metabolites during the day (open, cyan blue)/night (gray), and (j) heatmap showing metabolite concentrations in individual birds. The deep red and deep blue colors in the heatmap represent the maximum and minimum of the metabolite level, respectively.
In the annual life cycle of migratory birds, migrating and nonmigrating LHSs differ in energy metabolism. However, migration is about physiological ability to adequately enhance aerobic metabolism to efficiently mobilize/oxidize FA high-energy demands of migratory flight; the nonmigrating LHS is identified by lower daily energy expenditure with minimum daily differences in leptin, insulin, total serum proteins, and urea. In birds, plasma metabolite concentrations significantly vary among species and indicate clear differences in protein and lipid utilization and could be affected by diet. Notably, the seasonal and diurnal changes in the bird’s physiology vary with the LHS. The magnitude of biochemical changes in the captive caged bird reflects the actual changes in wild birds, but the concentration and extent of biochemical changes may vary. Furthermore, only male birds were used in the current study to minimize the metabolic difference in sexually dimorphic buntings. Our observation of daytime elevation of the narrow range of short-chain FAs, SCFA, monounsaturated MUFA, and polyunsaturated FAs, PUFA, in nonmigrating buntings (Figure 2a–d), viz., palmitate (16:0), oleate (18:1), linoleate (18:2), and linolenic acid linolenate (18:3), is in agreement with observations on nonmigrating sparrows; the latter was reported to selectively prefer mobilization of palmitate and linoleate. Among TCA cycle intermediates in nonmigratory buntings, night elevation of isocitrate might be related to a lower metabolic rate during the night that confounded NAD+/isocitrate dehydrogenase function, a regulatory enzyme.
within the tricarboxylic acid cycle (TCA), and fluctuations tending toward higher titers of oxalic acid in the night were suggestive of oxidation of glucose in night-fasted birds.24 Significantly higher concentrations of taurine (Figure 2a, j), a reactive carboxyl scavenger amino acid, in avian blood as compared to mammals were reported.25 Taurine, as an inhibitory neurotransmitter, has anticonvulsant and antianxiety properties, and its metabolic role is to promote insulin release. Therefore, taurine might have a putative role in mediating subtle metabolic advantages to songbirds in resisting glaucoma despite high glucose levels.56

Birds switch from the carbohydrate energy substrate to the lipid energy substrate during migration (Figure 3a–k). Glucose breakdown (glycolysis end product), that is, pyruvic acid, enters the tricarboxylic acid (TCA) cycle mediated by acetyl-CoA. Furthermore, acetic acid and acetoacetate, intermediates in lipolytic and protein substrates, levels were all-time high in migrating buntings, indicating high aerobic metabolism. Furthermore, during migration, many birds increase de novo lipogenesis activity to build fat stores, a process involving BMA1, a circadian clock protein.57 This might explain why daytime pyruvate levels are higher than night levels, in the present study. In support of this, lower hepatic daytime pdc mRNA levels (gene coding for the pyruvate dehydrogenase complex, PDC) confirm higher breakdown of pyruvate.7 On the one hand, lower pyruvate, yet persistently higher acetic acid levels, suggests FA oxidation as a source to the Krebs cycle, while on the other hand, we found hexanoic acid and butanoate, intermediates of β-oxidation of FA, to be significantly high during the night, an active phase of migration in migrating buntings. Biophysical studies involving sequential 13C NMR spectra compared two situations where intact hearts were supplied with perfusate containing either only palmitate (13C6) or palmitate coinfused with butyrate to every other carbon) or palmitate infused with butyrate to compare the rate-determining and competitive oxidation of PUFA versus both PUFA and SCFA. In other words, their results showed a correlation between palmitate transport, VPAL, and TCA cycle flux, VTCA. VTCA increased eight times more than VPAL in hearts oxidizing palmitate. In hearts oxidizing both palmitate and butyrate, VTCA is significantly increased. This is consistent with increased substrate availability (butyrate) which bypasses the regulatory proteins of palmitate oxidation.59 This happens because unlike palmitate, butyrate need not depend on FA transporters across the cell membrane or on carnitine shuttle (CPT1, CPT2, and CAT).59 Although, this kinetic analysis needs to be performed using the bird’s heart, it adds a new dimension in explaining birds’ increased aerobic metabolism during migration.59

Increased aerobic capacity is one among the determinants of successful migration. The oxidative capacity of muscle and the dynamics of fat storage in different parts of the body have a cumulative effect on the bird’s aerobic capacity during migration.31 The aerobic production of CO, H, O, and ATP from the lipolytic β-oxidation pathway in migrating birds is an important energy pathway. Lipolysis occurs simultaneously with lipogenesis. Increased lipogenesis with aerobic metabolism during migration is supported by elevated mRNA levels of fasn, a lipid synthetase enzyme, in migrating buntings, suggesting lipogenesis.18 We also found significantly increased concentration of an adipocyte-secreted FA synthesis intermediate, 16:0 carboxymidic acid, PEA, in migrating buntings. PEA, having anti-inflammatory and cannabinomimetic properties, and its metabolic role is to promote insulin release. Therefore, taurine might have a putative role in mediating subtle metabolic advantages to songbirds in resisting glaucoma despite high glucose levels.56

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Materials and Methods

The animal care and procedures adopted in this study were as per guidelines of the Institutional Animal Ethics Committee (IAEC) of Chaudhary Charan Singh University, Meerut. Adult birds were supplied with perfusate containing either only palmitate (13C6) or palmitate coinfused with butyrate to every other carbon) or palmitate infused with butyrate to compare the rate-determining and competitive oxidation of PUFA versus both PUFA and SCFA. In other words, their results showed a correlation between palmitate transport, VPAL, and TCA cycle flux, VTCA. VTCA increased eight times more than VPAL in hearts oxidizing palmitate. In hearts oxidizing both palmitate and butyrate, VTCA is significantly increased. This is consistent with increased substrate availability (butyrate) which bypasses the regulatory proteins of palmitate oxidation.59 This happens because unlike palmitate, butyrate need not depend on FA transporters across the cell membrane or on carnitine shuttle (CPT1, CPT2, and CAT).59 Although, this kinetic analysis needs to be performed using the bird’s heart, it adds a new dimension in explaining birds’ increased aerobic metabolism during migration.59

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male redheaded buntings (E. bruniceps) were acclimated to laboratory conditions while fed on cereal grains (Setaria italica) supplemented with gritted hard-boiled eggs and water, ad libitum. Post acclimatization, male birds (n = 19) were transferred to short days (8L:16D; short day: SD), that is, 8 h of light:16 h of dark and light intensity 1.55 W/m² during the light phase and 0.002 W/m² during the dark phase, and kept in well-aerated photoperiodic chambers (size: 1 m × 1 m × 1 m). During the time the birds were held under SDs, these exhibited diurnal behavior, that is, daytime activity and daytime feeding. Birds were reproducibly inactive under SDs, ruling out the possibility of any metabolic constrain due to breeding; this was supported by visual assessment of testis size. The earlier method, as discussed in Gupta et al. (2019), was adapted for the daily food consumption pattern and activity recordings.42 Ambient temperature was monitored using Easy Log USB, Lascar electronics Inc. PA, USA, and maintained constant at 22 °C. SBT was recorded twice (in the midday and midnight) 24 h using thermoscan (range 32−42.5 °C) at ~1 cm distance from the keel region skin surface exposed by gently blowing air.

**Experiment 1.** Male buntings (n = 7) were kept in individual cages (size: 45 cm × 30 cm × 30 cm) for 28 days in SDs (8L:16D), and blood samples (Figure 1b) were collected at two time points representing midday and midnight. The bird’s wing vein was punctured with a fine sterile needle, and blood droplets (yielding 100−250 µL volume) were gently collected into heparinized microhematocrit capillary tubes.

**Experiment 2.** Two groups of buntings (n = 6 each) were kept under SDs and long days (LD; 14L:10D). Birds held under LDs exhibited night activity behavior. Such a night flight behavior is photoinduced simulation of migration in wild conspecifics. Blood samples were drawn 4 hourly as per details in Experiment 1. Blood samples were kept at 25 °C for 0.5 h and centrifuged at 2000g at 4 °C for 10 min. Serum was collected and aliquoted in coded microcentrifuge tubes and stored at −80 °C until further analysis. Minimum two freeze thaw cycles were allowed before use of these materials. The blood sampling sequence was randomized, besides care was taken to avoid metabolic alteration due to physical stress, maintaining at least 36 h gap between two subsequent sample collections.

**QC and Randomization.** Following the recommended protocols, an equal volume of all study serum samples was pooled to prepare a quality control sample (QC).33,43 Coded samples were randomized using a web-based tool (www.randomizer.org) to process these for metabolite extraction and derivatization in batches followed by gas chromatography−mass spectrometry (GC−MS) data acquisition within 24 h of derivatization. To minimize operator biasness, we adopted a double blinding approach during serum sample collection and GC−MS data acquisition.

**Serum Processing and Derivatization.** In brief, the serum sample (50 µL) was thawed on ice, and freshly prepared isonicid solution (1 mg/mL, 10 µL) was added as an internal standard. Ice-cold methanol (800 µL) was mixed with the sample and vortexed for 30 s. The suspension was centrifuged at 15,000g for 10 min at 4 °C, and the supernatant was dried in a SpeedVac at 40 °C. The dried sample was treated with 2% methoxyamine HCl in the pyridine (MOX) reagent at 60 °C for 2 h followed by a silylation step with N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) at 60 °C for 1 h. After derivatization, the sample tube was centrifuged at 10,000g for 5 min, and the supernatant was transferred into a vial insert kept inside a 2 mL screw-capped glass vial.

**GC−MS Data Acquisition.** Using an automated sampler (MPS, Gerstel Germany), the derivatized test serum sample (1 µL) was injected using the splitless mode to an RTx-5 column (5% diphenyl, 95% dimethylpolysiloxane; 30 m × 0.25 mm × 0.25 µm; Restek USA) in a GC/TOF-MS (Pegasus 4, Leco, USA). Helium was used as carrier gas at a constant flow rate of 1 mL/min. The inlet temperature was fixed at 300 °C during injection, temperature gradients of 80−150 °C (ramp of 7 °C/min) and from 150 to 270 °C (ramp of 10 °C/min) with a hold time of 1 min between two ramps. After reaching the final temperature, a hold time of 15 min at the final temperature was maintained. The electron ionization (EI) mode was fixed at 70 eV to scan ions of 35−600 m/z ranges. The maximum scan speed was 20 Hz with a 230 s solvent delay. Ion source temperature was fixed at 240 °C, and data acquisition voltage was 1600. Sample introduction to data acquisition parameters in GC−MS was controlled through ChromaTOF software (Leco, USA), and the total run time was 2340 s per sample.

**Data Preprocessing and Peak Alignment.** Raw GC−MS data (.pek) files of all the study samples (n = 14) were preprocessed using ChromaTOF. Alignment of all GC−MS data files was carried out using the “Statistical Compare” feature of ChromaTOF. For peak picking, the peak width was set at 1 s and the signal/noise (S/N) threshold was 100. For tentative molecular feature identification, mainlib (2, 12, 961 spectra) and replib (30, 932 spectra) libraries from NIST (version 11.0) were used with a minimum similarity index of 750. Maximum retention time deference was set at 0.5 s, and for mass spectral match, minimum spectral similarity among aligned molecules was set at 600. Unsilylated molecules were removed from the data matrix manually. Aligned peak information was exported to the .csv format, and molecules absent in more than 50% of samples of at least one class (time point) were excluded from analysis. Missing values were imputed with half of the minimum value of study population, and the data matrix was normalized with the peak area representing the internal standard isoniazid. Univariate analysis in terms of the paired t-test (p < 0.05) was carried out using MetaboAnalyst. The selected important molecular list was taken for hierarchical clustering using Ward linkage, and the Euclidean cluster method was carried out to see the clustering pattern.

**Pathway and Statistical Analysis.** The retention time and fragmentation pattern of commercial standards of important metabolites were matched to establish the identity. To identify deregulated molecular pathways, identified molecules were used as the input list in MetaboAnalyst 3.0.45 The pathway library of Gallus gallus was used for pathway analysis. In behavioral study, Student’s t-test was used to compare total activity and food intake between different observations. One-way ANOVA followed by post-hoc Newman–Keuls was used for comparing body temperature at different time points. Significance was taken at p < 0.05. Data were plotted, and statistical analyses were performed using Prism GraphPad software (GraphPad ver. 5.0, San Diego, CA).

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Abbreviations

SD, short day; LD, long day; LHSs, life history stages; GC

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