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Physiology

The role of ketogenesis in the migratory fattening of the northern wheatear *Oenanthe oenanthe*

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The fuelling capacity of migratory birds and their ability to avoid health conditions derived from the subsequent fat overload are exceptional among vertebrates. In this work, we screen the expression of the genes involved in the production of ketone bodies (KB) in the liver of northern wheatears (*Oenanthe oenanthe*) during the development and resolution of migratory fattening. Thirteen genes were found to be regulated among the migratory stages. Based on the dynamics of gene expression, we concluded that KB play a versatile role in wheatears' energy metabolism homeostasis. The ketogenic pathway can adaptively: (i) provide carbon equivalents for lipogenesis, speeding up fuelling; (ii) replace glucose during long-distance flights using lipids as the substrate; (iii) act as a floodgate to avoid steatosis; and (iv) might provide a metabolic solution to defatting in captive birds.

1. Introduction

Migratory birds can gain weight at a rate of more than 10% of their lean body mass per day; some individuals double their weight—mostly through fat stores—before the onset of migration [1–4]. Hyperphagia, increased digestion efficiency, and specific metabolic adaptations are hypothesized to be involved in such a rapid gain of fat reserves [5]. In captivity, after a few weeks remaining 'very fat', birds spontaneously start defatting, returning to their pre-migratory weight [6–8]. Migratory birds' lipid metabolism and the mechanisms to counteract the adverse effects of obesity have evolved to fulfil the challenges of endurance flights [9–11]. Ketogenesis is a versatile process related to global energy homeostasis, linking lipids, carbohydrate and protein metabolism. Thus, ketogenesis is an attractive mechanism to study in relation to migratory fattening [12].

Ketone bodies (KB) are produced in the liver from fatty acids and certain amino acids, usually under low carbohydrate availability [13]. KB are secreted in the blood and used in peripheral tissues to replace glucose [14,15]. This means that the energy contained in fatty acids is exported to organs that can efficiently use KB as an energy source, such as the brain, kidneys and cardiac muscle [12,13,16].

To provide a continuous supply of glucose to the brain during long flights is challenging, because the liver glycogen reserves are limited and contribute to a small fraction of the total energy necessary for migration [10,15,17]. The amounts of liver glycogen stores have been shown to change seasonally in some bird species, correlating negatively with lipid deposits [18,19]. During

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migration, ketogenesis may provide an alternative to gluconeogenesis, supplying energy to the brain during flight or at poor stopover sites where fuel gain is not possible [20–22].

On the other hand, the role of ketogenesis has proved to be versatile under feeding conditions [23]. Under those circumstances, acetoacetate is routed to the cytoplasm as a substrate for lipogenesis [24,25]. In mammals, KB contribute between 54% and 80% of the carbon incorporated into fatty acids and sterols, respectively, depending on the feeding state [23,26].

Simultaneously, while fuelling, ketogenesis may act as a floodgate, keeping healthy levels of lipid stores and avoiding obesity-related complications like inflammation [27–30].

In birds, changes in plasma KB concentration have also been associated with their feeding and migratory status [31–35]. Therefore, analysis of the expression of genes involved in KB metabolism during the development and resolution of the migratory phenotype in birds will help to clarify their relevance with regard to migration.

In this work, we screened the expression of these genes by RNA-Seq in the liver of a long-distance migratory passerine, the northern wheatear (*Oenanthe oenanthe*), sampled in captivity at four different body mass stages (lean, fattening, fat, defatting) during the autumnal migratory season.

2. Material and methods

This paper is one of a series analysing the seasonal biochemical and molecular adaptations of *O. oenanthe* to migration. The procedures to collect the samples, isolate RNA and pre-process the sequence data described here are similar to those referred to in Frias-Soler *et al*. [36].

(a) Study species

The birds used in our study were raised in captivity at the Institute of Avian Research (Wilhelmshaven, Germany). To induce the development of the migratory fattening, all wheatears (except the 'lean birds' group, photoperiod 14 L : 10 D cycle) were subjected to a 12 L : 2 D cycle at 20 ± 1°C room temperature [37–39]. The body mass was recorded early in the morning every day, before the birds received food, a diet supplemented with mealworms [40]. The samples ($n = 15$) were collected based on the course of the body mass changes: (1) lean, $n = 4$ (one bird was sampled after completing the migratory cycle); (2) fattening, $n = 4$; (3) fat (kept constant body mass 'plateau'), $n = 3$; and (4) defatting, $n = 4$; all birds independently of their initial body mass, developed the migratory fattening (electronic supplementary material, table S1). Wheatears were euthanized following Guglielmo *et al*. [41]. The tissue was frozen in liquid nitrogen and stored at −80°C until RNA purification.

(b) RNA purification

For RNA purification, the GeneMATRIX Universal RNA Purification Kit (Roboklon GmbH, Berlin, Germany) and Roche (Roche GmbH, Germany) were used. We followed the general recommendations for RNA isolation of Sambrook *et al*. [42]. Polymerase chain reaction (PCR) targeting the mitochondrial cytochrome *b* gene was carried out, in order to check for the absence of DNA. Amplifications were undertaken in a 30 µl reaction volume containing 1.5 mM $MgCl_2$, 100 µM dNTPs, one unit of Taq DNA polymerase (Pharmacia Biotech, Munich, Germany), 4 µl of the RNA isolate and 5 pmol of each oligonucleotide [43]: mta1, CCCCCTACCAACATCTCAGCATGATGAAACTTG; and mtfr, CTAAGAAGGGTGGAGTCTTCAGTTTTTGGTT TACAAGAC. The PCR programme consisted of three steps: (i) denaturation at 94°C for 5 min; (ii) 35 cycles of denaturation at 94°C for 25 s, annealing at 50°C for 2 min, and extension at 72°C for 2 min; followed by (iii) a final extension at 72°C for 10 min. The RNA concentration, cleanliness and integrity were evaluated using standard spectrophotometric measurements and 1.5% agarose gel electrophoresis [42].

(c) Library preparation and sequencing

RNA samples (28 s/18 s ratio ≥ 1; RIN ≥ 6.5; A260/A280 ≥ 1.8) were sent to Beijing Genomics Institute (BGI), Hong Kong, China, for sequencing.

The mRNA molecules were purified from total RNA using oligo(dT)-attached magnetic beads, fragmented bands between 100 and 700 bp (major 200–300 bp) were selected. First-strand complementary DNA (cDNA) was generated using random hexamer-primed reverse transcription, followed by second-strand cDNA synthesis. The cDNA was subjected to end-repair and was then 3′ adenylated. Adapters were ligated to the ends of these 3′ adenylated cDNA fragments and the products were amplified by PCR. The amplicons were purified with Agencourt AMPure XP beads (paramagnetic bead-based technology). Library quality was validated using the Agilent Technologies 2100 bioanalyzer. The double-stranded PCR products were denatured, circularized and purified. The single-strand circular DNA (ssCir DNA) was formatted as the final library product for sequencing. The ssCir DNA library was amplified with phi29 to form a DNA nanoball (DNB), which generated around 300 copies of one single molecule. The DNBs were loaded into the patterned nanoarray and pair-end reads of approximately 100 bases were generated using the BGISEQ-500 sequencing system. For a detailed description of the protocol, see [44–46]. The raw sequence data (electronic supplementary material, table S3) can be found in the Sequence Read Archive (SRA) database [47].

(d) RNA-Seq raw data quality control and filtering

Inspection of the original data was conducted using the FastQC software v. 0.11.9 [48]. For filtering the fastq files (pair-ends), Trimmomatic-039 [49] was used, with the following parameters: -phred33, cropping the first 15 bp (HEADCROP:15), removing 5′ and 3′ bases with quality below 3 in a 4-base read wide sliding window, cutting when the average quality per base drops below 20 (LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20), and dropping reads shorter than 36 bp (MINLEN:36). The paired reads which matched between both corresponding fastq files—that is, forward and reverse—were employed for quantification.

(e) Mapping and quantification

Kallisto [50] was employed to index the reference transcriptome [51] and map the paired reads from the cleaned fastq files. The raw count matrix and the normalized estimation of gene abundance (transcripts per million, TPM) were generated for statistical analysis [52].

(f) Statistics

The R package DESeq2 [53] was used for differential expression analysis using shrunken log fold-changes values [54]; the rest of the parameters were kept as default. The adjusted *p*-value was calculated for each gene comparison, using the $p < 0.05$ threshold as the nominal value to reject the null hypothesis of no significant difference, or $\log_2$fold-change > 2 to consider a gene to be a candidate for being regulated. To correct *p*-values for multiple testing, the Benjamini–Hochberg method was used, as implemented in DESeq2. The comparisons were performed between all migratory conditions. The *p*-value frequency distribution was also explored in order to check for abnormal distributions.

The R programming language platform, v. 4.0.3, was employed for statistical analysis and plotting [55].

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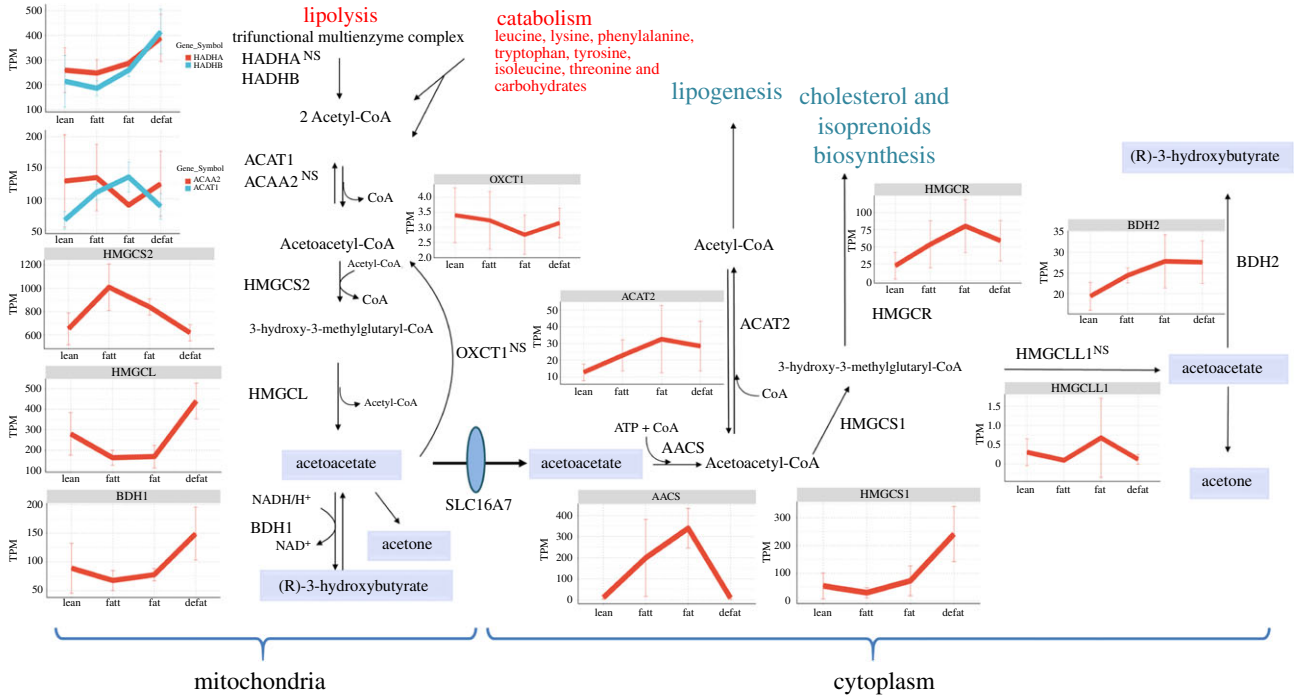

Figure 1. Interconnection among lipolysis, catabolism of amino acids and carbohydrates with ketogenesis and the biosynthesis of cholesterol, isoprenoids and fatty acids [12,13,15,25] in the liver of wheatears at different migratory stages.

3. Results and discussion

In this paper, we focus on discussing the metabolism of KB in the context of the migratory fattening and defatting processes of the northern wheatear.

Thirteen genes involved in ketogenesis were found to be regulated between body mass conditions in the livers of wheatears (electronic supplementary material, table S2). The expression of these genes in different migratory conditions shows how the pathway might contribute to fattening, the control of fat overload and the provision of a metabolic solution to defatting (figure 1).

(a) Role of ketone bodies during the feeding period

During the fattening and fat periods, the over-expression of HMGCS2 and the cytoplasmic AACS may favour acetoacetate usage—from amino acid and carbohydrate catabolism—for lipogenesis, thus contributing to fat storage, new tissue membrane generation and bile salt production [13,56].

The export of acetoacetate from the mitochondria to the cytoplasm may act as a parallel and expedited mechanism to the citrate–malate shuttle, in order to relocate carbon equivalents (four, instead of the two from acetyl-CoA) for lipogenesis [12,23]. Simultaneously the monocarboxylate transporter 2 (SLC16A7), responsible for transporting KB across the mitochondrial and cell membranes, is over-expressed in fat birds in comparison to lean wheatears. This may explain, at least partially, the rapid growth of fat deposits while fuelling.

(b) Role of the ketone bodies in defatting

During the defatting period, the genes involved in ketogenesis showed the following pattern:

(i) increased expression of HADH (fatty acid catabolism), and carnitine palmitoyltransferase 1A (CPT1A) and CPT2 (electronic supplementary material table S2), both of which are related to the mitochondrial uptake of long-chain fatty acids for their subsequent beta-oxidation. This suggests a sustained increment in lipolysis, meaning that the acetyl-CoA pool is derived mostly from fatty acids;

(ii) a decrement in the expression of HMGCS2 to its basal level (approx. 600 TPM, mean) but a simultaneous over-expression of HMGCL and BDH1, the genes responsible for the final two enzymatic steps of ketogenesis;

(iii) the monocarboxylate transporter 2 (SLC16A7) exhibited a sustained increment towards the defatting condition;

(iv) in the cytoplasm, AACS showed a decreased expression, contributing to acetoacetate accumulation and indicating a reduced or even aborted lipogenesis; and

(v) the over-expression of cytosolic HMGCS1 in defatting birds is puzzling, as its substrate (acetoacetyl-CoA) theoretically has a low concentration. The upregulation of HMGCS1 may be a compensatory reaction owing to the lower input of diet cholesterol, this is consistent with the expression of HMGCR.

HMGCS2 has been recognized as the rate-limiting step in the production of KB [15], and is regulated at the transcriptional level, by hormones, allosteric interactions and covalent modifications [57]. Nevertheless, our data showed that most of the genes involved in ketogenesis were under transcriptional control in wheatears. It is essential to consider that the basal expression of most of the studied genes was relatively high (except for AACS). This means that the production of KB is probably never aborted and the transcriptional regulation of the pathway may help to adaptively redirect the metabolic flux for lipogenesis or the ultimate production of KB.

During defatting, the over-expression of HMGCL and BDH1, the increment of the upstream pathway substrate

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(acetyl-CoA) and the reduced demand for acetoacetate in the cytoplasm owing to halted AACS transcription may displace the metabolic flux towards β-hydroxybutyrate production, in compensation for the reduced expression of HMGCS2.

The downregulation of HMGCS2 may be a consequence of fine-tuned regulation linked, for example, to the amount of fat reserves, signals associated with the switch between fat/defatting stages or metabolic status. Keeping HMGCS2 overexpressed for a long time may actually be compromising as fat reserves should be carefully depleted. Wheatears do not undergo this pronounced defatting process in the wild; there, fatty acids are valuable and are used simultaneously for muscular contraction [10,11,58] and KB production as a substitute for glucose [13,16]. Therefore, a reduction in the amount of transcribed HMGCS2 would facilitate control over the pathway's limiting step and, consequently, over the defatting process. Another point to consider is that, during defatting, KB enter the bloodstream in a higher concentration, a process that must be strictly controlled because of the risk of ketoacidosis [59,60].

Fatty acids are catabolized to acetoacetate and β-hydroxybutyrate to fuel different tissues, but acetoacetate can be non-enzymatically decarboxylated to acetone and can be eliminated by breathing [13]. The question therefore arises as to what magnitude wheatears exhale part of their lipid reserves.

Regarding the use of wheatears as an orthologous model for the study of obesity-related diseases, we can say that the defatting process resembles the characteristics of the 'ketogenic diet', based on low-sugar/high-fat meals. This diet has been efficiently used in humans for weight loss and to treat epilepsy [12,61–63].

4. Conclusion

The metabolism of KB and the putative adaptations needed to counteract the metabolic challenges of ketosis (e.g. ketoacidosis) deserve to be clarified in the context of avian migration. Ketogenesis seems to have an adaptive function during the development of the wheatear migratory phenotype, as follows: (i) facilitating lipogenesis during the accumulative phase through the use of carbon equivalents from amino acids and carbohydrate catabolism; and (ii) contributing to the control of steatosis and defatting by relocating the energy stored in fatty acids in peripheral tissues through the substitution of glucose. Further studies on the individual expression trends of ketogenesis-related enzymes, as well as their kinetic parameters and regulatory mechanisms, are necessary to understand their role in migratory fattening. This work supports previous findings, which have pointed at ketogenesis as a potential target for the management of obesity-related diseases in humans.

Ethics. The study was conducted according to the guidelines of the German Animal Welfare Act and approved by the Zweckverband Veterinäramt JadeWeser (42508-Te).

Data accessibility. The raw sequence data can be found in The Sequence Read Archive (SRA) database from the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/sra/) under the BioSample accessions: SAMN16177890-SAMN16177904 [47].

Authors' contributions. All authors have read, reviewed, edited and agreed to the published version of the manuscript. R.C.F.-S. wrote the paper, designed the methodology, sampled the birds, conducted bioinformatics analysis and interpreted the data. N.K. participated in sampling, the methodology design, reviewing and editing the manuscript. L.V.P. participated on the methodology design and conducting bioinformatics analysis. M.W. and F.B. are senior researchers and participated on the conceptualization of the manuscript, funding acquisition, supervision, reviewing and editing the manuscript. All authors agree to be accountable for all aspects of the work.

Competing interests. We declare we have no competing interests.

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