The insectivorous bat *Pipistrellus nathusii* uses a mixed-fuel strategy to power autumn migration

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In contrast to birds, bats are possibly limited in their capacity to use body fat as an energy source for long migrations. Here, we studied the fuel choice of migratory *Pipistrellus nathusii* (approximate weight: 8 g) by analysing the stable carbon isotope ratio ($\delta^{13}C_{V-PDB}$) of breath and potential energy sources. Breath $\delta^{13}C_{V-PDB}$ was intermediate between $\delta^{13}C_{V-PDB}$ of insect prey and adipocyte triacylglycerols, suggesting a mixed-fuel use of *P. nathusii* during autumn migration. To clarify the origin of oxidized fatty acids, we performed feeding experiments with captive *P. nathusii*. After an insect diet, bat breath was enriched in $^{13}$C relative to the bulk and fat portion of insects, but not deviating from the non-fat portion of insects, suggesting that bats oxidized exogenous proteins and carbohydrates, but not exogenous fatty acids. A feeding experiment with $^{13}$C-labelled substrates confirmed these findings. In conclusion, migratory *P. nathusii* oxidized dietary proteins directly from insects captured en route in combination with endogenous fatty acids from adipocytes, and replenished their body reserves by routing dietary fatty acids to their body reserves.

Keywords: chiroptera; energetics; migration; fuel choice; vertebrate flight

1. INTRODUCTION

Migratory birds and bats are known to move over long distances between a summer and winter habitat [1,2]. Although both taxa use aerial locomotion as an efficient mode of transport, migratory ranges differ greatly between birds and bats [3–5]. Many migratory birds cover long distances of many thousands of kilometres during their seasonal journeys [6], whereas only a few bats cover a few thousands of kilometres [7,8]. Along their journey, both taxa face high metabolic rates during sustained flights [9]. Consequently, migration is preceded by many physiological changes, such as hyperphagia and fat deposition (birds: [10–12]; bats: [13–15]). Triacylglycerols (TAGs) from adipocytes are the optimal energy source for powering endurance flights because of their high energy density [16,17]. Indeed, fatty acids originating from adipocyte TAG are the primary fuel for most migratory birds [12,18–20]. In contrast, even highly aerobic mammals depend more on intra-muscular fuels than on adipocyte fuel when exercising over long periods [21,22], which raises the question of whether bats are able to power endurance exercise solely via oxidation of fatty acids from adipocyte TAG. Also, bats may be constrained in the use of TAG during autumn migration, because they have to save adipocyte TAG for surviving the many months of hibernation. As an additional supply of energy, bats could hunt insects and oxidize exogenous nutrients directly [23,24], as recent observations of hunting in migratory bats suggest [25]. The additional use of exogenous nutrients would enable bats to save adipocyte TAG for hibernation. Yet excessive hunting during migration may impede bats from covering long distances, since foraging requires energetically costly and time-consuming aerial manoeuvres [26,27].

We studied the metabolic substrate use in migratory *Pipistrellus nathusii*. This insectivorous bat (approximate weight: 8 g) migrates seasonally from its northeastern range in Germany, Poland, Belarus, Fennoscandia, the Baltic States and Russia to the southwest of Europe where it hibernates in overground roosts such as in trees or buildings [28]. Earlier studies confirmed that banded bats cover up to approximately 2000 km (one way) along their annual migration [7,29], which is one of the longest recorded migratory movements for a bat worldwide [3]. To determine whether *P. nathusii* oxidize $^{13}$C-enriched proteins from insects that they hunt en route, or whether they oxidize $^{13}$C-depleted endogenous fatty acids from adipocyte TAG, we determined the source of oxidized substrates in migratory *P. nathusii* by measuring the stable carbon isotope ratios ($\delta^{13}C_{V-PDB}$) of exhaled breath; breath $\delta^{13}C_{V-PDB}$ matches closely $\delta^{13}C_{V-PDB}$ of the pool of oxidized substrates [30,31].

We hypothesized that migratory *P. nathusii* oxidize insect proteins or a mixture of exogenous insect proteins and endogenous fatty acids, because sedentary insectivorous bats are known to oxidize dietary nutrients or endogenous fatty acids from adipocytes according to
whether or not they have fed recently [24]. Accordingly, we predicted that $\delta^{13}C_{\text{VPDB}}$ of exhaled breath should be similar to $\delta^{13}C_{\text{VPDB}}$ of insects captured in the same habitat as bats. Alternatively, if migratory bats oxidize additionally or exclusively $^{13}$C-depleted fatty acids from adipocyte TAG, then $\delta^{13}C_{\text{VPDB}}$ of exhaled breath should be lower than $\delta^{13}C_{\text{VPDB}}$ of potential insect prey. To disentangle whether oxidized fatty acids originate from endogenous (adipose TAG from body reserves) or exogenous (insect fat) sources, we performed two feeding experiments. First, we measured $\delta^{13}C_{\text{VPDB}}$ of breath in resting P. nathusii after having fed several mealworms (larval stages of Tenebrio molitor, Coleoptera). We predicted that breath $\delta^{13}C_{\text{VPDB}}$ should be higher than $\delta^{13}C_{\text{VPDB}}$ of bulk mealworms and the fat portion of mealworms, but similar to the $\delta^{13}C_{\text{VPDB}}$ of the non-fat portion of mealworms, if bats oxidize exogenous proteins but not fatty acids. Second, we measured $\delta^{13}C_{\text{VPDB}}$ in breath collected from P. nathusii after having fed on a dose of $^{13}$C-labelled amino acids (glycine) or $^{13}$C-labelled fatty acids (palmitic acid). We calculated the cumulative oxidation of both substrates [32], and predicted that migratory P. nathusii oxidize the exogenous amino acid but not the fatty acid. Our experiments may shed new light on the physiological abilities and constraints of insectivorous bats when performing long-distance migration.

2. MATERIAL AND METHODS

Fieldwork was conducted at Pape Ornithological Station in Pape, Latvia (56°09' N 21°03' E) between 18 August and 5 September 2011. Migratory bats were captured between 21.00 and 2.00 h or dawn using a Helgoland funnel trap as described by Petersons [7].

(a) Experiment I: metabolic substrate use of migratory Pipistrellus nathusii

 Immediately after capture, we mechanically restrained P. nathusii by wrapping gauze bandage around their bodies. Then, bats were transferred singly into a plastic container (0.2 l volume; LockLock, iSi Deutschland GmbH, Solingen, Germany) that could be hermetically sealed. The container was equipped with an inlet through which CO$_2$-free air entered at a flow rate of 700 ml min$^{-1}$ [31]. At the opposite side of the container, we attached a needle (0.9 gauge; Braun, Melsungen, Germany). Once we closed the container with a bat inside, we flushed the container for 1 min with CO$_2$-free air. Afterwards, we stopped the flushing and allowed CO$_2$ to accumulate for 1.5 min. Then, we pierced the Teflon membrane of a Vacutainer (Labco, Buckinghamshire, UK) with the needle so that the air from inside the container (including the bat’s breath) was sucked into the Vacutainer. Accordingly, about 2.5–3 min passed between the capture of the flying bat and the collection of the breath sample. In theory, stress-related changes in metabolic fuel choice could have biased our results, because catecholamines and glucocorticoids respond quickly to stressors. However, plasma glucocorticoids have been shown to remain constant within a 3 min period following a stressor [33], and a previous model suggested that the hysteresis effect of a bat’s body bicarbonate pool is sufficiently slow to make stable carbon isotope ratios of breath representative of what happened a few minutes before [24]. After breath collection, animals were either brought to the station for further experiments or released. Vacutainers were shipped to the stable isotope laboratory of the Leibniz Institute for Zoo and Wildlife Research (IZW) where the stable carbon isotope ratio of CO$_2$ was analysed using a blind protocol within a maximum period of three weeks as described in earlier studies [24,27]. Samples were analysed together with a laboratory standard gas that we previously calibrated with the international $^{13}$C reference materials NBS 19 and L-SVEC. Ratios of $^{13}$C and $^{12}$C were expressed relative to the international standard (Vienna-PeeDee Belemnite) using the $\delta$ notation in parts per mille (%): $\delta^{13}C_{\text{VPDB}} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 10^3$, where $R$ is the ratio of heavy and light carbon isotopes ($^{13}$C/$^{12}$C) in the sample and the standard. Precision was always better than $\pm$ 0.14‰ (1 s.d.).

For endurance migration, insectivorous bats can use two major energy sources: $^{13}$C-enriched proteins (from captured insects) and $^{13}$C-depleted TAG (from captured insects or body reserves) [34,35]. Breath $\delta^{13}C_{\text{VPDB}}$ of fasting animals is on average approximately 3% lower than that of animals feeding on a protein diet, because fasting animals oxidize $^{13}$C-depleted fatty acids [31]. To compare breath $\delta^{13}C_{\text{VPDB}}$ with that of potential energy sources, we captured bats at the same time and at the same place where we captured bats. Insects were identified according to order and then morphotype. For shipment to the IZW, we stored insects in ethanol-filled plastic vials. In the laboratory, we collected two subsamples from each morphotype. We extracted fat from one of these samples by washing it with a 2:1 chloroform–methanol solution for 24 h. Afterwards, extracted and non-extracted samples were dried until constant mass in an oven at 50°C. We then filtered about 0.35 mg of dried samples in tin capsules. Loaded capsules were analysed as described in earlier studies [24,27]. Precision for bulk $\delta^{13}C_{\text{VPDB}}$ measurement was better than $\pm$ 0.10‰ (1 s.d.). To determine the $\delta^{13}$C of body fat in bats, we obtained fresh carcasses of P. nathusii from a project on the impact of windfarm facilities on European bat populations. All of these bats were killed by wind turbines in Germany between August and September. In a separate study, we modelled the geographical origin of these bats based on stable hydrogen isotope ratios of fur keratin and found that all most likely to have originated from the Baltic countries or Russia [36]. Thus, we used samples from these bats instead of sacrificing P. nathusii at our study site in Latvia. We dissected the bat carcasses and obtained white adipose tissue from the inter-scapular region for stable isotope analysis ($n = 6$). Fat samples were treated and analysed as described earlier.

During most nights, we captured bats during two periods (evening: 21.30–23.00 h; morning: 2.00–5.30 h). Consequently, we first tested whether breath $\delta^{13}C_{\text{VPDB}}$ of bats captured in the evening and in the morning differed by using an unpaired Student’s $t$-test with Welch correction. We then performed separate analyses for each activity period. In particular, we asked whether migratory P. nathusii captured during a given activity period oxidized exogenous nutrients (i.e. ingested insects) by comparing $\delta^{13}C_{\text{VPDB}}$ of bat breath with $\delta^{13}C_{\text{VPDB}}$ of local insects using a Mann–Whitney $U$-test. Additionally, we tested whether bats oxidize endogenous TAG by comparing $\delta^{13}C_{\text{VPDB}}$ of bat breath with $\delta^{13}C_{\text{VPDB}}$ of body fat using a Mann–Whitney $U$-test. All statistical tests were two-tailed, assuming an alpha-value of 5 per cent and using ISATYR v. 3 (GraphPad Software, Inc., San Diego, CA). We present values as mean $\pm$ 1 s.d. if not indicated otherwise.
(b) Experiment II: Pipistrellus nathusii feeding on mealworms

We fed eight *P. nathusii* (four males/four females) mealworms to see at what plateau breath $\delta^{13}C_{VPDB}$ would level off in relation to dietary $\delta^{13}C_{VPDB}$ when bats have access to exogenous nutrients. Bats were captured 1 day prior to the experiment and kept in a wooden box over 24 h. We used the same experimental set-up as previously described [24,27] to collect breath samples. From each bat, we collected a breath sample 100 min after the bat had started to feed on mealworms, because we expected breath $\delta^{13}C_{VPDB}$ to converge on $\delta^{13}C_{VPDB}$ of the oxidized substrate over this period in a bat of this size [24,31]. Before feeding and after taking the breath sample, we recorded the body mass of bats with an electronic balance (accuracy 0.01 g; Volckart, Germany). All bats were released at the site of capture. Breath samples were shipped to the stable isotope laboratory of the IZW and then analysed as described earlier.

We collected 50 mealworms to obtain reference values of $\delta^{13}C_{VPDB}$ from ingested insects. We extracted fat from mealworms using the same protocol as described earlier for free-ranging insects. We conducted stable isotope analyses for the bulk mealworm, the fat portion and the non-fat portion of the mealworm as described earlier. We tested whether mean breath $\delta^{13}C_{VPDB}$ differed from $\delta^{13}C_{VPDB}$ of bulk mealworms, the fat portion of mealworms and the non-fat portion of mealworms based on one-sample $t$-tests using Instat.

(c) Experiment III: Pipistrellus nathusii feeding on $^{13}$C-labelled substrates

During the course of our fieldwork, we captured 17 *P. nathusii* (5 males/12 females) using the Helgoland funnel trap and transferred these bats in pairs to our feeding experiment with labelled substrates. We fed 10 bats each with 5 mg $^{13}$C-labelled glycine (C1-glycine; Euriso-Top GmbH, Saarbrücken, Germany) and seven bats each with 5 mg $^{13}$C-labelled palmitic acid (C1-palmitic acid; Euriso-Top GmbH) to assess whether migratory bats oxidize either of the substrates, and, if so, how much of the orally fed dosage would be used for oxidation over the experimental period. Breath collection followed the procedure described for experiment II with the difference that breath samples were collected at times 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 75 and 90 min. Stable isotope ratios of breath were analysed as described earlier.

We used the delta notation of measurements into atom per cent according to Slater et al. [37] and followed McCue et al. [32] in calculating the cumulative amount of oxidized substrate based on excess atom fraction of $^{13}$C, $x^{13}(^{13}C)$, and extrapolated resting metabolic rates ($\dot{V}_{CO2}$). $x^{13}(^{13}C)$ was calculated as the difference between atom fraction of $^{13}$C, $x^{13}(^{13}C)$ in exhaled breath of fed and unfed animals. We used measurements of basal metabolic rate and thermal conductance in a closely related species (i.e. 6.6 g *P. pipistrellus* [9]) as the best estimate for $\dot{V}_{CO2}$ of *P. nathusii*. We converted published oxygen consumption rates of 0.202 ml O2 min$^{-1}$ into $\dot{V}_{CO2}$ assuming a respiratory quotient (RQ = $\dot{V}_{CO2}/\dot{V}_{O2}$) of 0.8 for protein digestion ($\dot{V}_{CO2}$ = 0.161 ml min$^{-1}$) and an RQ of 0.7 for fat digestion ($\dot{V}_{CO2}$ = 0.141 ml min$^{-1}$). To account for increased thermoregulatory costs in bats outside the thermo-neutral zone at 20°C, we multiplied these values by 1.4. We calculated the instantaneous rate of label oxidation (Ti, µg min$^{-1}$) following the Fick equation modified after equation (7) of McCue et al. [32] for substrate labelled with a single $^{13}$C per molecule. We used a bicarbonate retention factor of 86 per cent because previous studies in various mammal taxa consistently showed that bicarbonate retention was almost identical across taxa [38–41]. Any deviation between assumed and true bicarbonate retention factors may not affect the relative difference in our estimates of compound-specific oxidation rates because this constant applies to both calculations in the same way. Further, we have refrained from controlling for the loss of $^{13}$C in urea, because the suggested stoichiometric correction [32] may not accurately reflect the situation in mammals. The mentioned correction requires the following equation for urea-producing mammals: $k = [(C – 2N) \times 22.4] / M$, where $k$ is the volume of CO$_2$ (ml) produced for each milligram of tracer oxidized, 22.4 is the volume (l) of one mole, C and N the number of carbon and nitrogen atoms in the specific tracer molecule, respectively, and $M$ the mole mass of the tracer molecule. The factor 2 controls for the fact that twice the number of C are required to excrete a given number of N (this number reads 1.2 in the original equation for uric acid excreting birds [32]). In the present experiment, we have used glycine (2 C and 1 N), yielding a $k$-factor of 0 ml for the volume of CO$_2$ produced for each milligram of oxidized glycine. This contrasts with the fact that we were able to trace $^{13}$C in the exhaled breath of bats after they fed on glycine. Therefore, we assumed that all carbon atoms of the $^{13}$C-labelled substrates leave the body as CO$_2$ and not as urea. Accordingly, we have modified the above equation to: $k = [C \times 22.4] / M$. On this basis of assumption and the equations provided in McCue et al. [32], we then calculated the cumulative oxidation of $^{13}$C-labelled substrate (% of fed dose) over the 90 min experimental period.

In both experiments, we did not obtain sufficient CO$_2$ for stable isotope analysis from three individuals. Thus, we performed Friedman tests only with data of the remaining animals to test whether the excess atom fraction changes over time when bats are fed either glycine or palmitic acid. We compared the rate of oxidation using a Wilcoxon matched-pairs test with Instat, assuming an alpha-value of 5 per cent.

3. RESULTS

(a) Experiment I: metabolic substrate use of migratory Pipistrellus nathusii

We captured migratory *P. nathusii* during two activity peaks at night: in the late evening (21.30 until 23.00 h) and in the early morning hours (2.00 until 5.30 h; figure 1). Bats captured between 21.30 and 23.00 h weighed on average 7.6 ± 0.6 g and had an average breath $\delta^{13}C_{VPDB}$ of $-29.8 ± 1.1\%$ ($n = 20$). Body mass of bats captured between 2.00 and 5.30 h averaged 7.8 ± 0.8 g, which was not significantly different from those captured earlier (Student’s $t$-test: $t_{45} = 1.18, p = 0.246$). However, $\delta^{13}C_{VPDB}$ of breath collected from bats between 2.00 and 5.30 h was significantly lower than in those bats captured between 21.30 and 23.00 h ($\delta^{13}C_{VPDB} = -31.4 ± 2.4\%$, $n = 27$; $t_{25} = 2.7, p = 0.013$). All bats defaecated insect fragments when we collected breath samples or when we kept bats for a short period in separate linen bags. We captured nocturnal insects at our study site that belonged to three taxonomic groups: Diptera, Lepidoptera and Heteroptera. The large majority of all

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insects were nocturnal moths, and therefore we performed pair-wise $\delta^{13}$C$_{\text{PDB}}$ comparisons only in Lepidopterans ($n = 9$ morphotypes). In moths, $\delta^{13}$C$_{\text{PDB}}$ of the non-fat portion of organic tissues was more enriched in $^{13}$C by 1.5‰ ($-26.1 \pm 1.8$‰) compared with the total organic tissue ($-27.7 \pm 1.0$‰; paired Student’s $t$-test: $t_q = 4.4, p = 0.0023$). As the best proxy for the isotopic composition of the alternative endogenous fuel source in migratory $P.$ nathusii, we measured $\delta^{13}$C$_{\text{PDB}}$ of fat obtained from fresh bat carcasses at wind turbines [36]. Fat reserves of these migratory $P.$ nathusii were depleted in $^{13}$C ($-33.6 \pm 1.5$‰) in relation to potential insect prey at our study site in Latvia.

Breath $\delta^{13}$C$_{\text{PDB}}$ was lower than $\delta^{13}$C$_{\text{PDB}}$ of potential insect prey (both bulk or fat portion of samples), irrespective of whether bats were captured early or late in the night (bats captured in the evening versus insects: $U = 13, U' = 230, n_1 = 27, n_2 = 9, p < 0.0001$; bats captured in the morning versus insects: $U = 9, U' = 171, n_1 = 20, n_2 = 9, p < 0.0001$). The mean difference between $\delta^{13}$C$_{\text{PDB}}$ of bulk insects and breath $\delta^{13}$C$_{\text{PDB}}$ from bats captured in the evening equalled 2.2‰, and between $\delta^{13}$C$_{\text{PDB}}$ of bulk insects and breath $\delta^{13}$C$_{\text{PDB}}$ from bats captured in the morning equalled 3.7‰. Breath $\delta^{13}$C$_{\text{PDB}}$ of bats that were captured early in the night was higher than $\delta^{13}$C$_{\text{PDB}}$ of body fat ($U = 2, U' = 160, n_1 = 27, n_2 = 6, p < 0.0001$). However, breath $\delta^{13}$C$_{\text{PDB}}$ of bats captured in the morning was not significantly different from $\delta^{13}$C$_{\text{PDB}}$ of body fat ($U = 28, U' = 92, n_1 = 20, n_2 = 6, p = 0.0536$).

Figure 2. Difference in stable carbon isotope ratios between available nutrients ($\delta^{13}$C$_{\text{source}}$, ‰) and exhaled breath ($\delta^{13}$C$_{\text{breath}}$, ‰) of eight $P.$ nathusii that had repeatedly fed on mealworms over 100 min. Box margins indicate the 25 and 75 percentiles, and the solid lines within the boxes the median. The dashed line indicates when exhaled breath and nutrient have the same $\delta^{13}$C value. $p$-values indicate significant differences between nutrient $\delta^{13}$C$_{\text{PDB}}$ and breath $\delta^{13}$C$_{\text{PDB}}$.

(b) **Experiment II:** $P.$ nathusii feeding on mealworms

Before we started feeding, bats weighed $6.6 \pm 0.6$ g ($n = 8$ individuals). On average, we fed $9.3 \pm 2.5$ mealworms to each bat. Individual bats gained $0.5 \pm 0.2$ g body mass until the end of the experiment; an increase that is equivalent to more than 7 per cent of their initial body mass. At $t = 100$ min, $\delta^{13}$C$_{\text{PDB}}$ of bats averaged $-25.4 \pm 1.5$‰ (figure 2), which significantly deviated from the $\delta^{13}$C of bulk mealworms ($U = 1, U' = 63, n_1 = 8, n_2 = 8, p < 0.003$) and the fat portion of mealworm ($U = 0, U' = 48, n_1 = 8, n_2 = 6, p < 0.001$), but not from $\delta^{13}$C$_{\text{PDB}}$ of the non-fat portion of mealworm ($U = 22, U' = 42, n_1 = 8, n_2 = 8, p = 0.328$; figure 2).

(c) **Experiment III:** $P.$ nathusii feeding on $^{13}$C-labelled substrates

$\delta^{13}$C$_{\text{PDB}}$ of unfed bats averaged $-30.5 \pm 2.8$‰. A Friedman test showed that $\delta^{13}$C$_{\text{PDB}}$ did not change over the course of the experimental period when bats were fed $^{13}$C-labelled palmitic acid ($k = 7, l = 15, F = 17.6, p = 0.225$; figure 3a), whereas the atom fraction changed when bats were fed $^{13}$C-labelled glycine ($k = 4, l = 15, F = 27.4, p = 0.0172$; figure 3a). In bats fed $^{13}$C-labelled glycine, $\delta^{13}$C$_{\text{PDB}}$ increased after a few minutes following the feeding event (figure 3a). Peak
enrichments averaged 380.1 ± 75.8‰ at $t = 20$ min in bats fed $^{13}$C-labelled glycine. Afterwards, breath $\delta^{13}$CV-

PDB decreased to lower values of about 200‰ for the rest of the experimental period (figure 3a). Oxidation rates were always lower in bats fed $^{13}$C-labelled palmitic acid ($0.084 ± 0.016 \text{ nmol min}^{-1}$) than in bats fed $^{13}$C-labelled glycine ($18.2 ± 4.6 \text{ nmol min}^{-1}$; Wilcoxon matched pairs test: $T_{+} = 105$, $T_{-} = 0$; $n = 14$ pairs, $p < 0.001$). Within the experimental time, $P$. nathusii oxidized about 2 per cent of the ingested dose of $^{13}$C-labelled glycine, whereas it oxidized only about 0.01 per cent of the ingested dose of $^{13}$C-labelled palmitic acid (figure 3c,d).

4. DISCUSSION

To evaluate whether insectivorous bats face energetic constraints during migration, we studied what oxidative fuels $P$. nathusii choose to power their autumn migration. We found that $P$. nathusii used a combination of exogenous insect proteins and endogenous fatty acids derived from TAGs of adipocyte tissue to fuel the journeys to their wintering habitats. Our observation is based on three findings: first, we documented that all $P$. nathusii defecated insect fragments after being captured during migration, indicating that they hunted insects en route and suggesting that exogenous nutrients were available as an oxidative fuel to bats. Second, we found that the stable carbon isotope ratio ($\delta^{13}$CV-
PDB) was lower in exhaled breath of recently captured $P$. nathusii than in local insects but higher than in fat deposits typical for $P$. nathusii. These intermediate $\delta^{13}$CV-
PDB values of breath samples indicated a mixed use of $^{13}$C-enriched insect proteins and $^{13}$C-depleted TAGs. During the early morning hours (between 2.00 and 5.30 h), $\delta^{13}$CV-
PDB of bat breath converged on the isotopic composition of adipocyte TAG, suggesting that oxidation of fatty acids from adipocytes became more important later at night. Third, we demonstrated in feeding experiments that $P$. nathusii oxidized the protein but not the fat portion of an insect diet, or the amino acids and not the fatty acids in experiments with $^{13}$C-labelled substrates. In contrast to migratory $P$. nathusii, non-migratory insectivorous bats, such as Noctilio albiventris, oxidized both the non-fat and the fat portion of recently ingested mealworms [24].

The finding that migratory $P$. nathusii use a mixed-fuel strategy (combined use of exogenous amino acids and endogenous fatty acids as an oxidative fuel) for autumn migration is novel, and most relevant for our understanding of the ecology and conservation of migratory insectivorous bats. First of all, migratory insectivorous bats seem to depend on continuous supplies of insects along their migratory route and not only at stopover sites. Indeed, it is unclear whether bats need to make stopovers for refuelling given their ability to hunt insects while migrating towards their wintering habitats. Second, fat deposits are essential for migratory insectivorous bats, not only for hibernation but also for powering the strenuous migratory flight, at least partly. Oxidation of fatty acids from adipocyte TAG seems to become increasingly important as an energy supply with decreasing insect abundance during the second half of a night (this study), and possibly also during cold nights when air-borne insects are scarce. An excessive depletion of fat stores may even hamper insectivorous bats from continuing their migration or to save sufficient fat deposits for surviving several months of hibernation. Thus, a high availability of nocturnal insects, and consequently an uninterrupted sequence of intact ecosystems, seems to be crucial for the successful migration of insectivorous bats. Consequently, conservation efforts in support of migratory bats should not necessarily focus on one or a few stopover sites, as is the case in conservation plans supporting migratory birds, but rather on intact and continuous migratory corridors.

Our findings are also interesting from a physiological perspective. Migratory $P$. nathusii routed exogenous TAG (presumably) to adipose tissues instead of oxidizing them immediately. This nutrient routing seems to be highly efficient given that dietary fatty acids were not oxidized at all in our feeding experiment with $^{13}$C-labelled substrate. Thus, aerial refuelling has two aspects in...
P. nathusii: the immediate oxidation of exogenous proteins and carbohydrates as a power supply, and the routing of exogenous fatty acids to adipose tissue for later use as an energy supply for either migration (when insects get scarce) or for hibernation. Interestingly, bats oxidized glycine at a lower rate than house sparrows (Passer domesticus). The underlying cause for this difference is yet unclear, but given the many differences between the studied vespertilionid bat and passerine bird (e.g. in body mass, phylogeny, origin, feeding habit, migration status), this difference is not surprising. In summary, aerial refuelling seems to represent a highly efficient migration strategy for insectivorous bats to minimize time spent along the migratory route. A similar strategy has already been suggested for migratory nectar-feeding bats, such as Leptonycteris curasaoae, that benefit from nectar provided by agave and cactus plants along a migratory corridor from the southern United States to Mexico [42]. The efficacy of such an aerial refuelling strategy is even increased in bats when migrants use torpor during daytime rest, a strategy that has been recently defined for Chiroptera as ‘torpor-assisted migration’ [43].

Migratory birds and bats of the Northern Hemisphere respond physiologically to the same adverse environmental conditions, namely reduced food availability and decreasing ambient temperatures. Insectivorous bats that migrate at night cannot forage for insects because of the limited ability to use vision at night to locate and hunt insects. Also, they are incapable of extended periods of torpor, let alone hibernation [3]. Therefore, migratory birds are probably forced to move towards areas with a sufficient and continuous supply of food and acceptable climatic conditions. In contrast, insectivorous bats of the temperate zone do not have to take the risk of an extended migration, because they are capable of surviving the cold winter by hibernation. This seems to be a better migratory strategy for P. nathusii than continuing to more southerly wintering habitats. Possibly, a mixed-fuel strategy in autumn could power even longer migrations. Yet it is uncertain whether bats could also benefit from a mixed-fuel strategy when migrating backwards to their breeding site, since they could also experience an extended migration strategy when migrating backwards to their breeding status), this difference is not surprising. In summary, aerial refuelling seems to represent a highly efficient migration strategy for insectivorous bats to minimize time spent along the migratory route. A similar strategy has already been suggested for migratory nectar-feeding bats, such as Leptonycteris curasaoae, that benefit from nectar provided by agave and cactus plants along a migratory corridor from the southern United States to Mexico [42]. The efficacy of such an aerial refuelling strategy is even increased in bats when migrants use torpor during daytime rest, a strategy that has been recently defined for Chiroptera as ‘torpor-assisted migration’ [43].

We conclude that insectivorous migratory bats, such as P. nathusii, use a mixed-fuel strategy to power autumn migration. This strategy contrasts with the TAG-fuel strategy of most temperate zone birds [10–12]. The mixed-fuel strategy allows bats to oxidize nutrients immediately en route and to refuel their fat deposits at the same time. Insectivorous bats of the temperate zone have found a unique way of supplying their migration with energy by oxidizing simultaneously exogenous proteins and endogenous fatty acids derived from adipocyte TAG.

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