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RESEARCH ARTICLE

Oxidation of linoleic and palmitic acid in pre-hibernating and hibernating common noctule bats revealed by ^{13}C breath testing

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ABSTRACT

Mammals fuel hibernation by oxidizing saturated and unsaturated fatty acids from triacylglycerols in adipocytes, yet the relative importance of these two categories as an oxidative fuel may change during hibernation. We studied the selective use of fatty acids as an oxidative fuel in noctule bats (*Nyctalus noctula*). Pre-hibernating noctule bats that were fed ^{13}C-enriched linoleic acid (LA) showed 12 times higher tracer oxidation rates compared with conspecifics fed ^{13}C-enriched palmitic acid (PA). After this experiment, we supplemented the diet of bats with the same fatty acids on five subsequent days to enrich their fat depots with the respective tracer. We then compared the excess ^{13}C enrichment (excess atom percentage, APE) in breath of bats for torpor and arousal events during early and late hibernation. We observed higher APE values in breath of bats fed ^{13}C-enriched LA than in bats fed ^{13}C-enriched PA for both states (torpor and arousal), and also for both periods. Thus, hibernating bats selectively oxidized endogenous LA instead of PA, probably because of faster transportation rates of polyunsaturated fatty acids compared with saturated fatty acids. We did not observe changes in APE values in the breath of torpid animals between early and late hibernation. Skin temperature of torpid animals increased by 0.7°C between early and late hibernation in bats fed PA, whereas it decreased by –0.8°C in bats fed LA, highlighting that endogenous LA may fulfil two functions when available in excess: serving as an oxidative fuel and supporting cell membrane functionality.

KEY WORDS: Hibernation, Fatty acid metabolism, Bats

INTRODUCTION

Many mammals overcome resource-poor and adverse winter periods by hibernating, a prolonged torpor during which animals reduce their body temperature (Lyman et al., 1982). Hibernation may last for several months, depending on, e.g., climatic conditions, taxon and life-history stages (Geiser and Ruf, 1995; Geiser, 1998). Mammals prepare for hibernation by hyperphagia and altered activity patterns, which ultimately lead to increased fat stores (Speakman and Rowland, 1999; Florant and Healy, 2012). During hibernation, mammals then oxidize fatty acids (FA) from their adipocytes to fuel torpor and also arousal events. FA are aliphatic chains of 12 to 24 carbon atoms associated with a carboxyl acid group. They play key roles in cellular structure and energy metabolism as phospholipids and triacylglycerols, respectively. FA may be saturated (SFA) or unsaturated, the latter including one or more double bonds in monounsaturated (MUFA) and polyunsaturated FA (PUFA), respectively. PUFA are derived from the diet in mammals, as mammals cannot synthesize these molecules *de novo*. PUFA are thought to be especially important for low metabolic rates at hypothermic body temperatures of hibernators (Florant, 1998; Munro and Thomas, 2004; Ruf and Arnold, 2008; Gerson et al., 2008; Arnold et al., 2015) and also for high metabolic rates (Price, 2010). Usually PUFA come as two forms in animals. Those with the first double bond located at the third carbon position – counting from the methyl (ω) end of the aliphatic chain – are called ω3, whereas those with the first double bond located at the sixth carbon position are called ω6. For hibernating mammals, it was argued that ω6 such as linoleic acid, and the ratio between ω6 and ω3, are essential to ensure correct function of cardiomyocytes (heart muscle cells) (Florant, 1998; Munro and Thomas, 2004; Gerson et al., 2008; Ruf and Arnold, 2008). This expectation is based on the observation that high ω6/ω3 ratios in cardiomyocytes enable torpid mammals to lower their body temperature without compromising important membrane functions such as the activity of the sarcoplasmic reticulum Ca^{+2} ATPase (SERCA; Ruf and Arnold, 2008). Overly high cytosolic concentrations of Ca^{2+} are thought to increase the risk for arrhythmia and cardiac arrest during hypothermia (Ruf and Arnold, 2008). Contrary to ω6, ω3 seem to suppress SERCA activity, yet high ω3 concentrations in phospholipids support pathways that promote transport of Mg^{2+} and Ca^{2+} ions across the membrane of the sarcoplasmic reticulum in heart muscles (Ruf and Arnold, 2008). On the one hand, PUFA are known to produce reactive oxygen species during peroxidation (Carey et al., 2000; Staples and Brown, 2008; but see Gerson et al., 2008). Thus, hibernators should be prudent in using PUFA as an oxidative fuel. On the other hand, elevated PUFA levels may increase the metabolic performance of animals, e.g. when bats raise their metabolic rate from torpor to flight during arousal events, due to the high transport rates of PUFA en route for oxidation (Price, 2010; Price et al., 2014). Therefore, hibernators should preferentially use PUFA during arousal events, which may ultimately lead to a depletion of PUFA in the adipocytes.

Here, we asked if common noctule bats (*Nyctalus noctula*), an insectivorous bat species from the Eurasian temperate zone, use SFA and PUFA selectively during the pre-hibernation and hibernation period. We fed pre-hibernating *N. noctula* one of three diets: ^{13}C-labelled palmitic acid (SFA group), ^{13}C-labelled linoleic acid (PUFA group) or a control diet. Then we measured ^{13}C enrichment of exhaled breath to estimate the tracer-specific oxidation rate. Linoleic acid is a PUFA (18:2) known to be enriched in adipocytes of hyperphagous animals during the pre-hibernation period and having a beneficial effect on hibernation

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Table 1. Comparison of peak metabolic rate, peak enrichment of ^{13}C in exhaled breath and peak skin temperature during the 40 min after onset of the arousal, and total arousal duration in individuals of the SFA and PUFA groups between early and late hibernation

	SFA group	PUFA group
Early hibernation		
Metabolic rate (ml min^{-1})	5.9±0.8	5.4±2.02
Peak enrichment (APE)	0.003±0.003	0.074±0.023
Peak skin temperature (°C)	32.0±3.6	31.7±2.4
Arousal duration (min)	61.9±26.4	38.9±14.1
Late hibernation		
Metabolic rate (ml min^{-1})	5.5±2.0	6.6±0.6
Peak enrichment (APE)	0.011±0.009	0.070±0.021
Peak skin temperature (°C)	29.1±9.4	32.9±1.9
Arousal duration (min)	45.2±10.8	38.0±12.3

Data are means±s.d.

(Munro and Thomas, 2004; Ruf and Arnold, 2008; Kolomiytseva, 2011). Palmitic acid is a SFA (16:0) and is commonly found in adipocytes of animals (Wells et al., 1965; Falkenstein et al., 2001; Souci et al., 2008; Mustonen et al., 2009). We predicted that dietary SFA such as palmitic acid would be oxidized more readily than dietary PUFA such as linoleic acid to save the latter for the coming hibernation period. Accordingly, we expected higher oxidation rates for palmitic than for linoleic acid in pre-hibernating bats. Alternatively, we expected higher oxidation rates of PUFA compared with SFA, because of the faster transportation of PUFA to the sites of oxidation in mitochondria that may support the metabolic capacity of bats (Price, 2010; Price et al., 2014).

After this experiment, we enriched adipocytes of pre-hibernating bats with ^{13}C-labelled palmitic or linoleic acid by supplementing the normal mealworm diet of bats with one of the ^{13}C-labelled fatty acids. We then measured the ^{13}C enrichment of bats when hibernating and when arousing from hibernation. We hypothesized that endogenous SFA should be oxidized preferentially during arousal events, when body temperature is higher, to save endogenous PUFA for the following torpor bouts. Accordingly, we expected a lower oxidation rate for PUFA than for SFA during hibernation but an increased PUFA oxidation rate towards the end of hibernation when SFA depots become depleted. Alternatively, PUFA are oxidized preferentially during arousal events because of their faster transportation to mitochondria, which may cause a gradual depletion of PUFA during the course of hibernation. This may lead to an increased use of SFA during torpor and arousal events towards the end of hibernation.

As we were not able to estimate the amount of labelled fat stores, we used the ^{13}C enrichment in the exhaled breath as a proxy for the relative extent to which animals use endogenous palmitic or linoleic acid as an oxidative fuel throughout the hibernation period. If hibernating bats oxidize PUFA in a prudent way because of detrimental by-products, we expected animals enriched in ^{13}C-labelled linoleic acid to show low ^{13}C enrichments in early hibernation, but high ^{13}C enrichments in late hibernation. We expected the opposite pattern if torpid bats preferentially oxidize PUFA. Furthermore, if hibernating bats oxidize SFA preferentially, we expected for animals enriched in ^{13}C-labelled palmitic acid to show high excess ^{13}C enrichments in early hibernation but low excess ^{13}C enrichments in late hibernation. We expected the same pattern for arousal events, i.e. a prudent use of linoleic acid as an oxidative fuel for arousal events in early but not in late hibernation, and a preferential use of palmitic acid as an oxidative fuel for arousal events in early but not in late hibernation. If arousal events depend more strongly on oxidation of linoleic acid because of the more efficient transportation of this fatty acid, we expected higher oxidation rates of linoleic acid during arousal in early hibernation, but lower oxidation rates of linoleic acid during arousal in late hibernation owing to depletion of this fatty acid towards the end of hibernation.

MATERIALS AND METHODS

Experiments were conducted at the Leibniz Institute for Zoo and Wildlife Research (IZW) in Berlin, Germany, under permission IC 113-G 0340/12 of the State Office of Health and Social Affairs Berlin. We captured 16 adult male *N. noctula* (Schreber 1774) from a municipal park in October 2013 under licence OA-AS/G/1009 of the Senatsverwaltung Berlin. We measured body mass (Pesola balance, Baar, Switzerland; 1 g accuracy) and forearm length (conventional handheld balances, 1 mm accuracy). Bats were maintained in captivity until mid-April 2014 when they were released after a final positive health check at the site of capture.

Pre-hibernation period

Before hibernation, bats were housed separately in cages at an ambient temperature of 20°C. Individual cages were made out of transparent plastic with a size of 28×21×10 cm^3. The rear of the cages was made out of metal mesh to facilitate exchange of air. We placed a piece of mesh on the cage ceiling so that bats could hold on to it. Additionally, a piece of cotton towel was attached to the ceiling of each cage for bats to rest. In addition, we offered *ad libitum* water in a small bowl. In order to maintain a relatively high humidity, we placed two water containers on the floor of the maintenance room. On a daily basis, we removed excreta from cages and fed each bat with about 50 mealworms. Eventually, bats learned to feed on their own from little bowls containing mealworms that were placed at the bottom of their cages. Bats were assigned randomly to groups according to one of the experimental diets: linoleic acid (PUFA group), palmitic acid (SFA group), or control diet.

Experimental protocol

Between 16 and 24 October 2013, bats were fed a single bolus of the corresponding tracer at the onset of night when bats had not yet been fed with their normal mealworm diet. Bats of the PUFA group (N=5 individuals) were each fed with 5 mg ^{13}C-enriched linoleic acid (99.9%, Cambridge Isotope Laboratories, Tewksbury, MA, USA) mixed in 50 µl conventional rapeseed oil. Rapeseed oil contains mostly monounsaturated fatty acids. Bats of the SFA group (N=6) were each fed with 5 mg ^{13}C-enriched palmitic acid (99.9%, Cambridge Isotope Laboratories) mixed in 50 µl rapeseed oil, and individuals of the control group (N=5 individuals) were each fed 50 µl of rapeseed oil only. Immediately after having received their bolus meal, bats were placed singly in a respirometry chamber (850 ml, Lock & Lock; QVC Handel, Düsseldorf, Germany) for measuring changes in excess ^{13}C enrichment in exhaled CO_2. We were unaware of the fatty acid composition of the bat diet prior to the captive period and also of the exact requirements of bats for essential fatty acids. Therefore, it was difficult to assess whether or not bats were in need of linoleic acid. We recommend that future studies should extend the captive period and control and analyse the fatty acid composition of bats to better judge the specific nutritional status of experimental animals with respect to essential fatty acids.

The respirometry chamber had a 10 by 12 cm mesh (Casanet 6.3 mesh width; Hornbach-Baumarkt-AG, Bornheim, Germany) at one wall where the bat could rest. The chamber was placed in a climatized animal container to control the ambient temperature at

30°C, which is within the thermal neutral zone of *N. noctula*. A pump (Maxima-R; Hagen Deutschland, Holm, Germany) pushed air at a rate of 1 litre^{-1} min^{-1} through the respirometric set-up. Inlet air was scrubbed of CO_2 by absorbents (NaOH: Carl Roth, Karlsruhe, Germany; Spherabsorb: Carl Roth) and water was removed by water absorbers. Water was removed from the air leaving the respirometry chamber (silica blue gel; Sigma-Aldrich, Munich, Germany) and then flow rate was measured and controlled (EL-Flow mass flow controller; Bronkhorst High-Tech, Kamen, Germany). Afterwards, air went into the stable cavity ring-down spectrometer (model 912-0003; Los Gatos Research, Mountain View, CA, USA). Stable carbon isotope ratios ($\delta^{13}C$) in exhaled CO_2 are provided in the delta notation (Slater et al., 2001) as parts per mille deviation from the international carbon standard (Vienna Pee Dee Belemnite) unless stated otherwise.

Before and after each measurement, background CO_2 concentrations were measured for testing air tightness of the set-up and for correcting possible offsets in CO_2 concentrations. We calibrated the system by measuring $\delta^{13}C$ of a reference gas (8% CO_2, Linde, Munich, Germany) with known $\delta^{13}C$ values (−4.1‰). For this, 15 ml of the gas were injected into the batch inlet of the isotope analyser. The reference gas was measured before and after each measurement as well as every hour after the onset of measurements. We corrected for instrumental offset and drift by fitting a polynomial regression on $\delta^{13}C$ values of the reference gas and by subtracting extrapolated values from measured values.

Before and after each experiment, we measured core body temperature with a rectal thermometer (GTH 1170 digital thermometer; Greisinger Electronics, Regenstauf, Germany) and body weight of the experimental animal. We ensured that bats were non-torpid at the onset of measurements based on the measurement of core body temperature. We considered a body temperature higher than 34°C as evidence for normothermy. Then we fed the tracer (or carrier liquid in case of control animals) and transferred bats immediately afterwards into the respirometric chamber. We measured $\delta^{13}C$ values and CO_2 concentrations for 5 h post-feeding. After the measurements, bats were fed and put back in their maintenance cages.

Calculation of tracer oxidation rates

$\delta^{13}C$ values were converted to atom fractions ($x^{13}C$ or AP) according to Slater et al. (2001). To correct for background isotopic enrichment of unfed animals, excess atom percentage (APE) was calculated according to $APE=AP_e-AP_b$; with AP_b and AP_e representing background and enriched values of unfed and fed animals, respectively. We used the median $\delta^{13}C$ value of breath of control animals at a given time as background values. The instantaneous tracer oxidation rate (TOR; nmol min^{-1}) was calculated using a modified Fick equation (McCue et al., 2010, 2017; Voigt et al., 2012; Hatle et al., 2017). For each sampling event, we calculated $\dot{V}_{CO_2}$ (ml min^{-1}) by multiplying the combined concentrations of $^{13}CO_2$ and $^{12}CO_2$ (ppmv) with the flow-through rate in the chamber using eqn (10.5) from Lighton (2008). All values are presented for standard temperature and pressure conditions.

Statistical analysis

We compared peak TOR and corresponding times between group PUFA and group SFA based on a Mann–Whitney *U*-test. The control group was not included in the comparisons as calculation of peak TOR used data from control animals as baseline values for APE. Data of time corresponding with the peak TOR was normally distributed and therefore we performed a Welch's two-sample *t*-test. We chose a 5% level of significance for all tests.

Collection and analysis of faecal matter

In order to test if tracer molecules were excreted instead of oxidized or assimilated, we collected faecal pellets from bats at 0 and 24 h after the feeding event. Dried faecal samples were homogenized, loaded into tin capsules (OEA Laboratories, Callington, Cornwall, UK) and then analysed in an elemental analyser (Flash EA 1112; Thermo Fisher Scientific, Bremen, Germany) connected to an isotope ratio mass spectrometer (IRMS; Delta V Advantage; Thermo Fisher Scientific) via a Conflo III interface (Thermo Fisher Scientific). We included a laboratory protein standard in our analysis to check for instrument offset and drift. Repeated measurements of laboratory standard gave an accuracy of <0.1‰ (one standard deviation) for $\delta^{13}C$ values. Differences in $\delta^{13}C$ values of faecal samples were compared between bats of PUFA and SFA groups. As data were not normally distributed, we used Kruskal–Wallis rank sum tests, followed by Wilcoxon signed rank tests, assuming a 5% level of significance.

Enrichment period

After the end of feeding trials during the pre-hibernation period, we implanted temperature-sensitive transponders (BioMedic Data Systems, Seaford, DE, USA) into bats for measuring skin temperature. Transponders were placed below the dorsal skin anterior of the scapulae and adjacent to the assumed site of brown adipose tissue. Transponders were calibrated by the manufacturer to an average body temperature of 25°C. Measured temperatures that deviated from the 25°C calibration temperature were offset corrected according to calibration curves that we established by measuring temperatures when transponders were placed in a water bath with a defined water temperature ranging between 5 and 40°C (Sonorex Super RK 103H, Bandelin Electronic, Berlin, Germany). For calibration, temperatures of the water bath were maintained at 5°C intermediate steps and calibration curves were then established for each transponder.

On each of five subsequent days, we fed bats a 5 mg bolus of their corresponding tracer before animals were fed their normal batch of mealworms. Repeated feeding of tracers to animals was performed to enrich body tissues, particularly adipocytes, with fatty acids carrying the ^{13}C marker. After each feeding event, we collected faeces from the cage floor to test if all marker molecules were absorbed. Faecal matter was analysed as described before.

In order to test if some of the tracer molecules were excreted instead of oxidized or assimilated, we used a Friedman test to monitor significant changes in ^{13}C enrichment in faecel pellets. We then used a Kruskal–Wallis test to check if ^{13}C enrichments varied across groups, followed by pairwise Mann–Whitney *U*-tests. All tests were calculated with SYSTAT. All parameters are given as medians unless started otherwise.

Hibernation period

For triggering the hibernation state of bats, cages were placed in refrigerators (Bioscape, Castrop-Rauxel, Germany; and Hanseatic, Otto, Hamburg, Germany) with the temperature set to 15–18°C and relative humidity to 60–65%. Ambient temperature and humidity in the refrigerators were chosen according to mean ambient conditions during October at our field site (about 16°C and 64%, German meteorological service, Offenbach, Germany). Temperature and humidity inside the refrigerator were checked daily from the outside using a thermo- and hygrometer (Hagen Deutschland).

Journal of Experimental Biology

Journal of Experimental Biology (2018) 221, jeb168096. doi:10.1242/jeb.168096

Starting on 11 November, we reduced the inside temperature of the refrigerator to about 6°C. Ambient humidity was set to constant 95%. These ambient conditions mimicked conditions in natural hibernacula of *N. noctula* (Arlettaz et al., 2000) and were thus maintained until the end of experiments on 16 April. In addition, we reduced the daily food supply by increasing the feeding intervals between 11 and 30 November, i.e. for a one-week period, bats were only fed every second day and during the subsequent two weeks bats were fed only every fourth day. Weighing and cage cleaning were scheduled accordingly. Starting on 1 December, we stopped feeding bats. Starting from this date, we weighed bats or cleaned cages only every two or three weeks in order to avoid waking up hibernating bats.

Respirometric measurements during arousal events

Respirometric measurements were carried out twice in hibernating bats: in January (early hibernation) and in March (late hibernation). All respirometric measurements were performed during the daytime and only with one bat at a time. During measurements, we monitored the body temperature of the focal bat with the implanted temperature-sensitive transponder that was read remotely using a stationary reading device (VSP-7005, BioMedic Data Systems). To avoid simultaneous arousal of all hibernating bats when a single bat was retrieved for respirometric measurements, we held bats scheduled to be measured during the next three days in a separate refrigerator (Hanseatic BCD-310CS; Otto) in a different room using the same set of ambient temperature and humidity conditions.

For respirometric measurements of bats arousing from hibernation, we used the same set-up as for the pre-hibernation measurements with the exception that temperature was set to 6°C in the climatized container. Before and after each respirometric measurement, we measured $\delta^{13}C$ values of the reference gas to control for instrumental offset and drift. We transferred experimental bats from the refrigerator to the respirometric chamber, which usually triggered the arousal of bats. Then we started respirometric measurements for periods that included a full arousal to euthermic conditions and re-entry into torpor (usually 2–3 h). Measurements were stopped with the onset of torpor, i.e. when CO_2 concentrations dropped below 200 p.p.m. or body temperatures below 20°C. After re-entering torpor, bats were kept in the respirometric chamber until body temperature reached levels around 6°C to ensure torpid conditions for measurement of $\delta^{13}C$ values under torpor conditions. This took approximately 1 h.

Respirometric measurements during torpor

Following the measurement of $\delta^{13}C$ values in arousing bats and re-entering of torpor, we performed respirometric measurements of torpid bats. As the cavity ring-down spectrometer was not able to measure $\delta^{13}C$ values at low CO_2 concentrations (below 100 p.p.m.), we trapped the air inside the respirometric chamber for 20 min to let CO_2 accumulate. We extracted air from the chamber using syringes and performed six repeated measurements of $\delta^{13}C$ in bat breath. We then calculated the median $\delta^{13}C$ value. We corrected for instrumental offset by measuring $\delta^{13}C$ values of the reference gas. After these measurements, bats were returned to their maintenance cage.

Statistical analysis

As we were not able calculate TOR in hibernating or arousing bats, we used peak APE in exhaled breath as a proxy for the relative use of a given tracer molecule in hibernating (torpor and arousal) and post-hibernating bats. APE values were not normally distributed. Power transformation of raw data did not result in normally distributed data. Therefore, we used a Friedman test to compare peak APE values in aroused bats during early and late hibernation and post-hibernation. Furthermore, we used a Wilcoxon test to compare peak APE values in torpid bats during early and late hibernation. We chose a 5% level of significance for all tests. Data are available from the Dryad digital repository (https://doi.org/10.5061/dryad.hr744).

RESULTS

Pre-hibernation period

Before the onset of experiments, noctule bats weighed on average 30.2±1.5 g (mean±s.d.; range: 28–32.5 g). Average body masses of bats did not differ among individuals of the three groups (Kruskal–Wallis rank sum test: χ^2=1.5, d.f.=2, *P*=0.472). Peak tracer oxidation rate (TOR) was recorded at 102±27 min post-feeding in group PUFA and at 101±13 min post-feeding in group SFA, which was not significantly different (Welch's two sample *t*-test: *t*=0.08, d.f.=7.94, *P*=0.94). Maximal TOR averaged 19.4±7.7 nmol min^{-1} for linoleic acid in group PUFA and 1.6±0.4 nmol min^{-1} for palmitic acid in group SFA (Fig. 1). TOR decreased to 5.3±1.7 nmol min^{-1} in group PUFA and 0.27±0.17 nmol min^{-1} in group SFA until the end of measurements at around 3.5 h. Peak TOR was about 12 times higher in the PUFA group than in the SFA group (Mann–Whitney *U*-test: *U*=30, *P*=0.004) in pre-hibernating noctule bats.

Seven out of 16 bats defaecated after the feeding experiments. $\delta^{13}C$ values averaged –22.0±2.2‰ (*N*=3), −20.4±1.3 ‰ (*N*=3) and 1533‰ (*N*=1) in the control group, group SFA and group PUFA, respectively. Twenty-four hours post-feeding, we obtained faecal samples from 13 bats. Variation of $\delta^{13}C$ values was small in animals of the control group (−25.3±1.6‰; *N*=4), but large in animals of groups SFA and PUFA, ranging between −25.6 and 785‰ in group SFA (*n*=5) and between −19.7 and 353‰ in group PUFA (*N*=4). $\delta^{13}C$ values of pellets were not different between bats of the SFA and PUFA groups (Mann–Whitney *U*-test: *U*=14, *P*=0.327).

Enrichment period

During each of the 5 days of the enrichment period, we obtained faecal pellets from all bats. Again, we observed a small variation of $\delta^{13}C$ values in pellets obtained from animals of the control group (range: −28.0 to −21.7‰) and a large variation of $\delta^{13}C$ values in pellets obtained from bats of the PUFA and SFA groups (ranges: PUFA group, −18.1 to 1630‰; SFA group, −0.2 to 661‰). Over the 5 day enrichment period, $\delta^{13}C$ values of faecal samples changed by 0.1‰ in the control group (Friedman test: χ^2=10.2, d.f.=4, *P*=0.037) but not in the PUFA group (χ^2=4.8, d.f.=4, *P*=0.308) or in the SFA group (χ^2=8.1, d.f.=4, *P*=0.087). According to a Kruskal–Wallis test, median $\delta^{13}C$ values of faeces differed among animals of the three groups (Kruskal–Wallis test: χ^2=10, d.f.=2, *P*=0.007; median values: control $\delta^{13}C$=−26.7‰, PUFA $\delta^{13}C$=71.9‰, SFA $\delta^{13}C$=53.4‰). Animals of the PUFA group defaecated pellets that were more enriched in ^{13}C by about 100‰ than those of animals from the control group (Mann–Whitney *U*-test: *U*=0, *P*=0.014), yet $\delta^{13}C$ values of faeces did not differ between animals of the PUFA and SFA groups (Mann–Whitney *U*-test: *U*=11, *P*=0.465). Faecal pellets of bats from the SFA group were more enriched in ^{13}C by 80‰ compared with those of bats from the control group (Mann–Whitney *U*-test: *U*=0, *P*=0.011). Body mass of bats increased until the onset of hibernation, i.e. when we triggered torpor behaviour by gradually reducing the amount of offered food, irrespective of group membership (one-way ANOVA: $F_{2,14}$=0.444, *P*=0.651). When entering the hibernation period, body mass of bats averaged 35.0±1.7 g.

Journal of Experimental Biology (2018) 221, jeb168096. doi:10.1242/jeb.168096

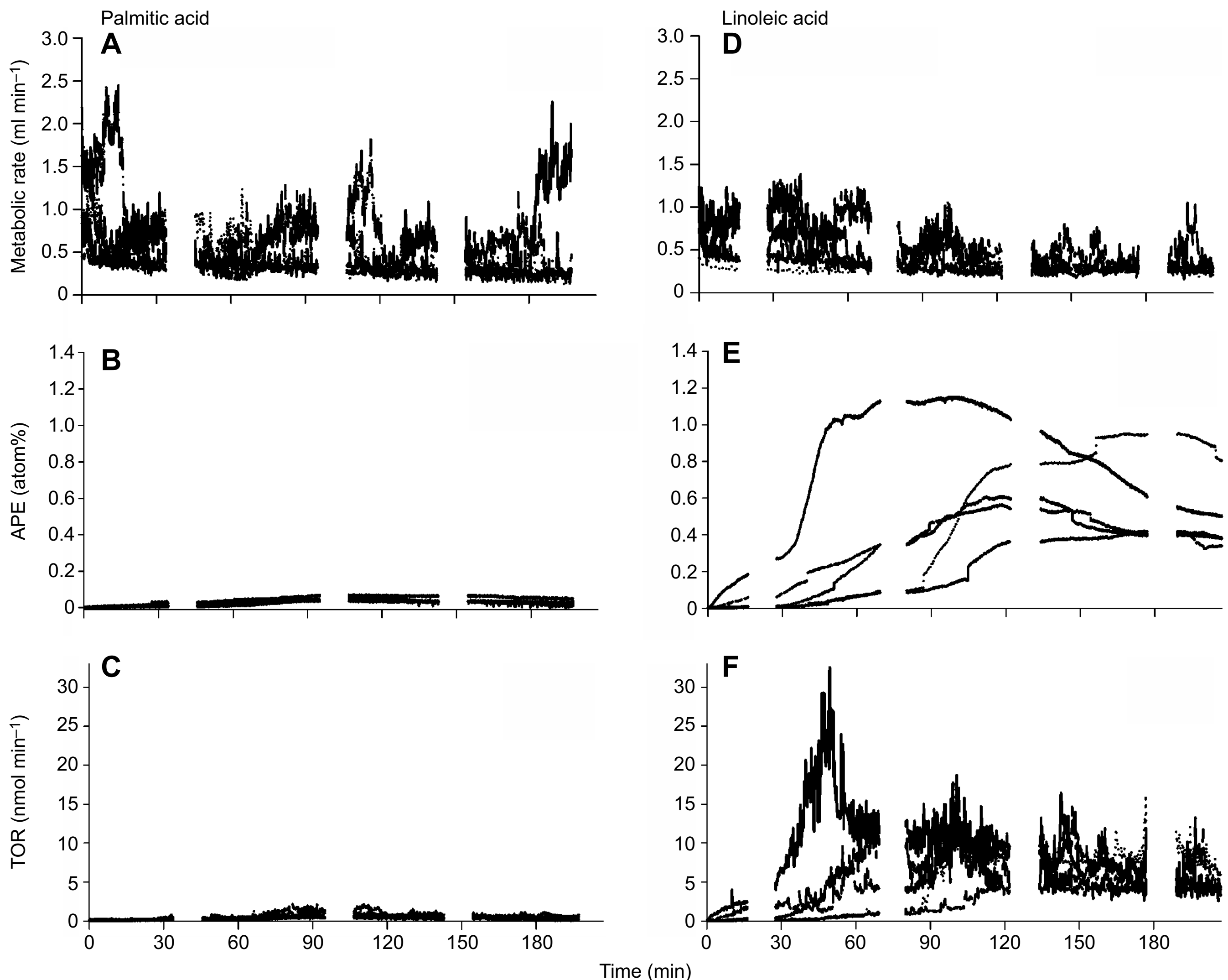

Fig. 1. Changes in CO_2 production rate, ^{13}C excess enrichment and tracer oxidation rate in relation to time elapsed after feeding the tracer to noctule bats (*Nyctalus noctula*) during the pre-hibernation period. (A,D) CO_2 production (metabolic) rate; (B,E) ^{13}C excess enrichment (APE); (C,F) tracer oxidation rate (TOR). The graphs on the left (A–C) show data from bats fed ^{13}C-enriched palmitic acid (SFA group), and the graphs on the right (D–F) show data from bats fed with ^{13}C-enriched linoleic acid (PUFA group). Intermission periods without data indicate calibration intervals.

Hibernation period

Over the period of hibernation, bats lost on average 8.9±1.4 g of body mass and weighed 26.3±1.8 g in early April. Mass loss did not vary among treatment groups (Kruskal–Wallis rank sum test: χ^2=0.316, d.f.=2, *P*=0.854). During hibernation, we observed that one bat of the control group had $\delta^{13}C$ values in exhaled breath higher than those during the pre-hibernation experiments. Therefore, we assumed that this bat was contaminated with some of the ^{13}C-labelled fatty acid by a contaminated feeding device during the enrichment period. Therefore, this animal was excluded from further analysis.

Torpor

Skin temperature of torpid animals of the SFA group increased from early to late hibernation by 0.7±0.6°C, averaging 5.7±1.1°C during early hibernation and 6.4±0.9°C during late hibernation (Wilcoxon test: *P*=0.046). In contrast, skin temperature decreased in bats of the PUFA group from early to late hibernation by 0.8±0.5°C; averaging 6.8±0.7°C during early hibernation and 6.0±0.9°C during late hibernation (Wilcoxon test: *P*=0.042), yet for each specific hibernation period, treatment groups did not differ in skin temperature (early: *U*=23.5, *P*=0.118; late: *U*=21.0, *P*=0.273). Torpor duration during experiments did not vary between experimental groups or between early and late hibernation (experimental groups, Mann–Whitney *U*-test: *U*=24, *P*=0.10; early–late hibernation, Wilcoxon test, *P*=0.213). We did not observe changes in the excess enrichment of ^{13}C (APE) in exhaled breath within the two groups between early and late hibernation (SFA: −0.002±0.003 atom%; PUFA: 0.056±0.024 atom%; Wilcoxon test: SFA: *N*=6 pairs, *P*=0.50; PUFA: *N*=5 pairs, *P*=0.99). APE values of torpid bats were higher for PUFA-fed animals compared with corresponding values from SFA-fed animals in both periods (Mann–Whitney *U*-test, early hibernation: *U*=0, *P*=0.006; late hibernation: *U*=0, *P*=0.006). APE values close to zero in the SFA-treated group indicated no use of ^{13}C-enriched palmitic acid as an oxidative fuel, whereas APE values of animals in the PUFA group indicated some extent of use of ^{13}C-enriched linoleic acid as an oxidative fuel in torpid bats.

Journal of Experimental Biology

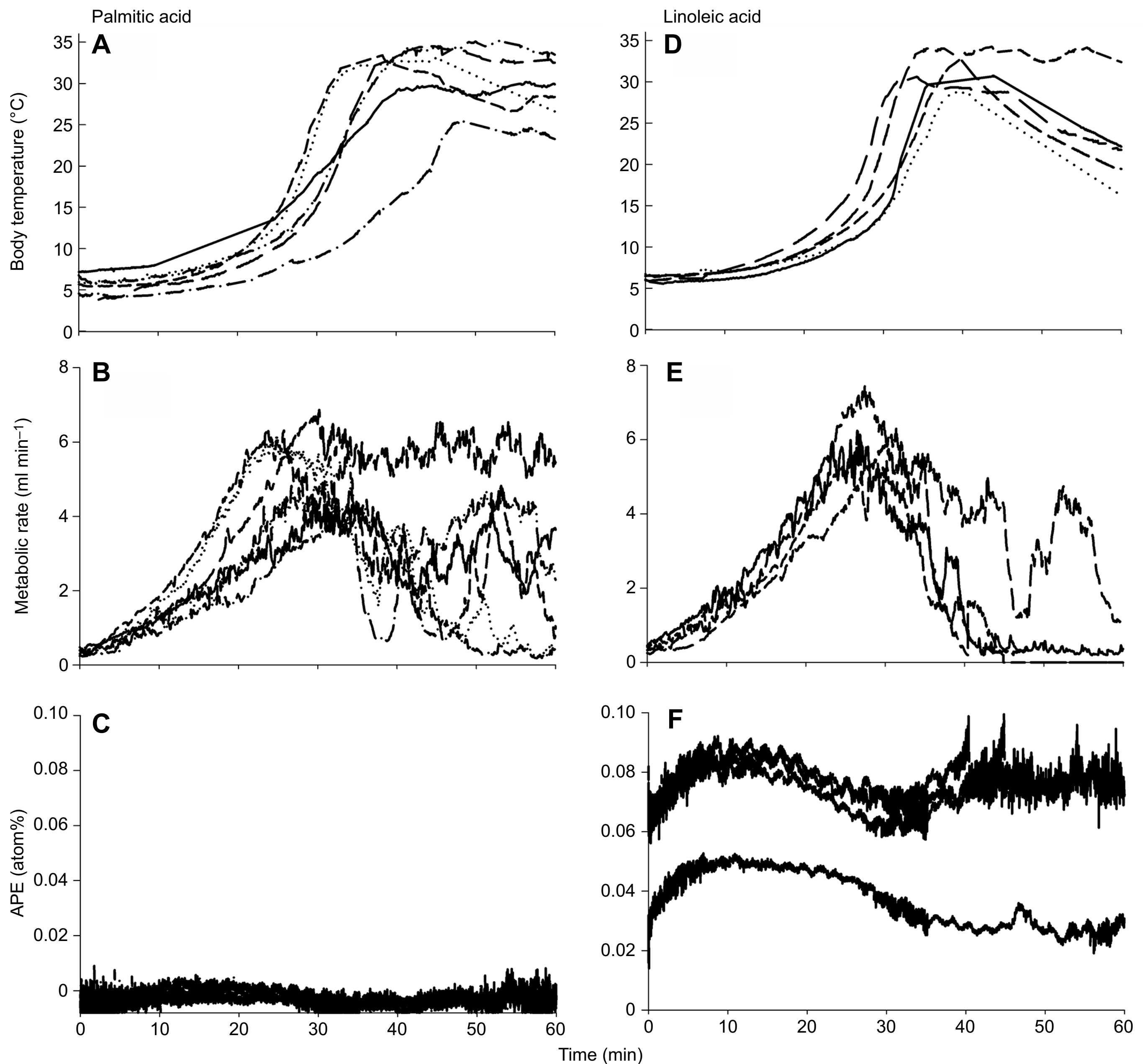

Fig. 2. Changes in body temperature, CO_2 production rate and ^{13}C excess enrichment in relation to time elapsed after onset of arousal during early hibernation in January. (A,D) Body temperature; (B,E) CO_2 production (metabolic) rate; (C,F) ^{13}C excess enrichment (APE). The graphs on the left (A-C) show data from bats of the SFA group, and the graphs on the right (D–F) show data from bats of the PUFA group.

Arousal during early and late hibernation

During early hibernation, bats arouse from torpor within about 40 min (Fig. 2A,D). Peak metabolic rates were recorded at 25.2±1.0 and 28.9±2.0 min after the onset of the arousal event in the PUFA and SFA group, respectively (Fig. 2B,E). Peak APE values during the first 40 min following the onset of arousal were about 25 times higher in bats of the PUFA group (0.074±0.023 atom%) than in those of the SFA group (0.003±0.003 atom%; Mann–Whitney *U*-test: U=0, P<0.009; Fig. 2C,F, Table 1). During late hibernation, one bat from the PUFA group could not be stimulated to arouse from torpor. Therefore, it was excluded from further analysis. During late hibernation, bats also took about 40 min to arouse from hibernation (Fig. 3A,D). During this 40 min period, peak metabolic rates were observed after 27.0±0.6 min after the onset of arousal in individuals of the PUFA group and 24.1±0.01 min in individuals of the SFA group (Fig. 3B,E). Peak APE values during this period averaged 0.070±0.021 atom% in the PUFA group and 0.011±0.009 atom% in the SFA group (Mann–Whitney *U*-test: U=0, P=0.009, Fig. 3C,F, Table 1). Peak APE values were about six times higher in bats of the PUFA group compared with those of the SFA group, yet they did not change from early to late hibernation in the SFA group (Wilcoxon test: P=0.893) or in the PUFA group (Wilcoxon test: P=0.686) for aroused bats.

DISCUSSION

We studied the selective use of SFA and PUFA during the pre-hibernation and hibernation period in the Eurasian common noctule bat (*N. noctula*). When feeding a dosage of the corresponding fatty

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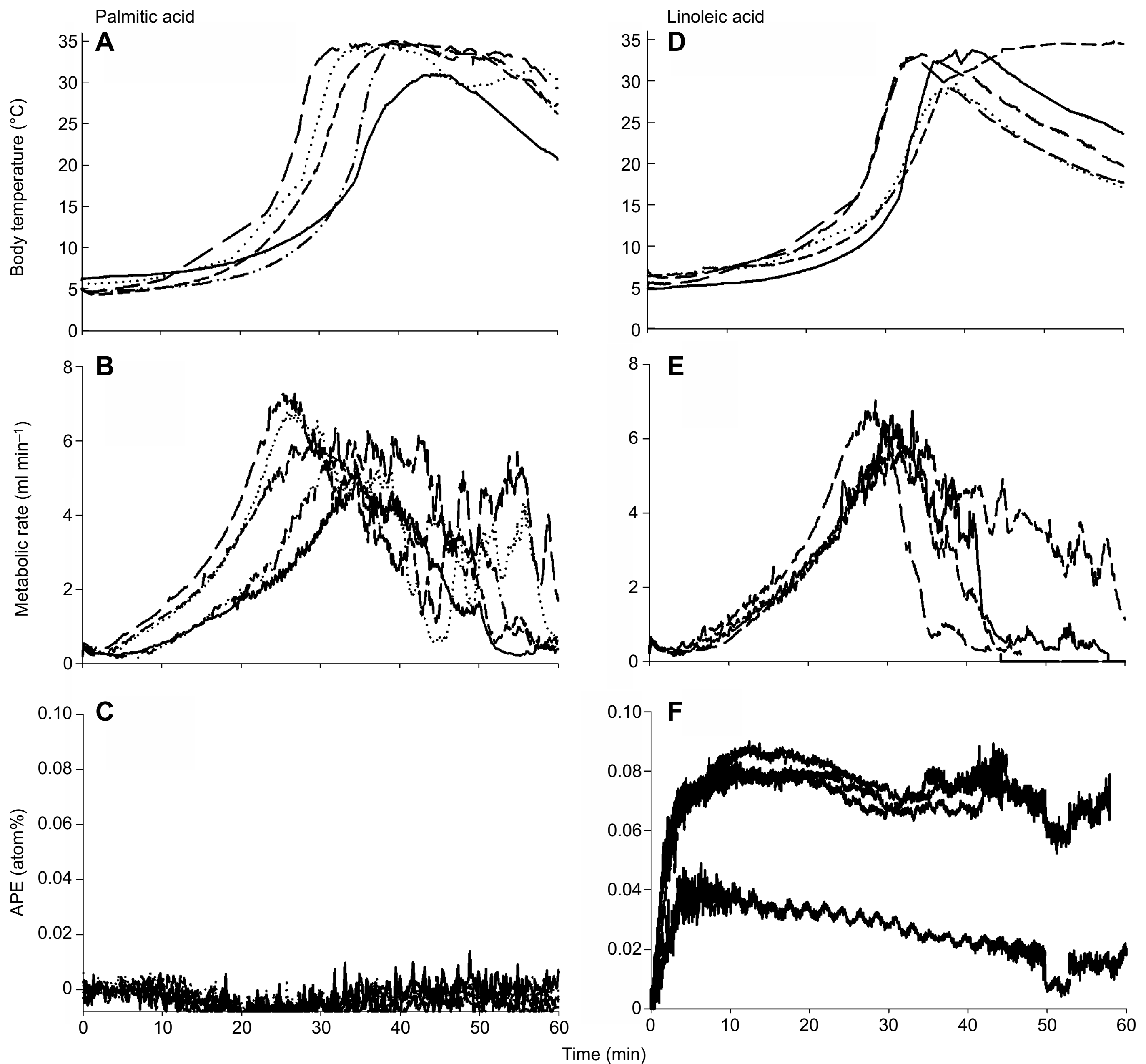

Fig. 3. Changes in body temperature, CO_2 production rate and ^{13}C excess enrichment in relation to time elapsed after onset of arousal during late hibernation in March. (A,D) Body temperature; (B,E) CO_2 production (metabolic) rate; (C,F) ^{13}C excess enrichment (APE). The graphs on the left (A-C) show data from bats of the SFA group, and and the graphs on the right (D–F) show data from bats of the PUFA group.

acid, pre-hibernating noctule bats were more likely to oxidize linoleic acid but not palmitic acid. Before hibernation, we further enriched the body of bats with one of the tracer molecules and monitored to what extent bats would use the endogenous fatty acids as an oxidative fuel. We found that hibernating bats fed with ^{13}C-enriched linoleic acid exhibited higher ^{13}C enrichments in their breath compared with bats that were fed ^{13}C-enriched palmitic acid, indicating that hibernating bats were more likely to use endogenous linoleic acid as an oxidative fuel than palmitic acid. We did not observe significant changes of ^{13}C enrichment in the breath of bats between early and late hibernation periods, irrespective of the specific fatty acid that they were fed. Furthermore, we noted that bats that were fed ^{13}C-enriched linoleic acid prior to hibernation were able to reduce their body temperature from early to late hibernation, whereas conspecifics that were fed ^{13}C-enriched palmitic acid increased their body temperature from early to late hibernation.

Pre-hibernation period

During the pre-hibernation period, bats were fed one of two ^{13}C-enriched fatty acids (either palmitic or linoleic acid). We then monitored the excess ^{13}C enrichment in exhaled breath (APE) using a laser spectroscope. Peak tracer oxidation rates (TOR) were measured for both compounds at about 100 min post-feeding, which was covering a longer period compared with previous experiments in, e.g., house sparrows (*Passer domesticus*), which were fed with palmitic acid (about 75 min) (McCue et al., 2010). TOR for palmitic acid was similar in noctule bats and laboratory mice, ranging between 0.5 and 1.6 nmol min^{-1} (this study; see also

Journal of Experimental Biology

Journal of Experimental Biology (2018) 221, jeb168096. doi:10.1242/jeb.168096

McCue et al., 2017), yet migratory *Pipistrellus nathusii* that were fed palmitic acid had lower TOR than noctule bats after the migration period (this study; see also Voigt et al., 2012), indicating that phylogeny, body size or season (migration versus pre-hibernation) may affect TOR in mammals in general and in bats in particular. In common noctule bats, TOR was 12 times higher for linoleic acid than for palmitic acid. Unfortunately, TOR of linoleic acid has not been measured before in any other bat or any other bird. Therefore, we cannot compare TOR values of linoleic acid within bats or across taxa. However, the observed difference in TOR between the two fatty acids highlight that pre-hibernating bats might prefer linoleic acid over palmitic acid as an oxidative fuel, probably as a result of higher transportation rates from the digestive tract to the sites of oxidation in mitochondria (Price, 2010).

We measured stable carbon isotope ratios in faecal pellets that were collected shortly after animals consumed the dosage of ^{13}C-enriched fatty acid and also at 24 h post-feeding. For both periods, we detected in some (but not all) pellets elevated ^{13}C enrichments compared with the control group. Overall, the isotopic variation of faecal pellets between individuals was large for bats when fed ^{13}C-enriched fatty acids. We infer from this large variation that either pellets got contaminated with some tracer, or that some of the tracer may have been excreted. The fact that the enrichment of pellets with ^{13}C was inconsistent within groups argues for the contamination hypothesis. Because of the uncontrolled loss of some of the tracer, we may have overestimated TOR values in pre-hibernating bats. Most previous studies did not assess a potential tracer loss in excreta and thus established TOR values might be overestimated in some of these studies as well when tracer molecules got lost during the feeding of animals.

Enrichment period

We enriched fat depots of bats with the respective ^{13}C-enriched tracer molecule by feeding bats meals of the corresponding tracer molecule over five subsequent days. Bats of the control group produced faecal pellets with background δ^{13}C values. In contrast, conspecifics that were fed one of the ^{13}C-enriched fatty acids showed consistently higher δ^{13}C values in their pellets, indicating that they accumulated some of the tracer in the digestive tract and excreted it over time. Surprisingly, δ^{13}C values did not change over the 5 days that bats were fed tracer molecules, as could be expected when the digestive tract of bats becomes enriched with the marker. In support of our earlier conclusion that elevated values might have originated from contamination, we observed both background and elevated δ^{13}C values in pellets of bats of experimental groups. We speculated that some of the tracer particles dropped from the mouth of bats when they groomed themselves after a meal. We assume that collected faecal pellets became contaminated with such particles, leading to ^{13}C enrichment of pellets above baseline values.

Hibernation period

Our study shows that bats preferentially use linoleic acid rather than palmitic acid as an oxidative fuel during both torpor and arousal events. Interestingly, increased oxidation rates of linoleic acids preceded the rise of skin body temperature and metabolic rates during arousal events. We thus argue that the preferential use of PUFA is caused by faster transportation rates of PUFA than SFA in torpid bats (Price, 2010, Price et al., 2014). By-products of PUFA peroxidation, such as reactive oxygen species, may not be of strong relevance in this process. Linoleic acids were preferred as an oxidative fuel even though PUFA are more valuable for hibernating bats to secure cell function in hypothermic conditions than SFA (Florant, 1998; Munro and Thomas, 2004; Gerson et al., 2008; Ruf and Arnold, 2008; Arnold et al., 2015). Overall, our findings are consistent with the hypothesis that PUFA are preferentially oxidized because of their better transportation rates compared with SFA (Price, 2010; Price et al., 2014). In addition, fatty acids such as linoleic acid might be more readily available as an oxidative fuel than saturated fatty acids, as they are more fluid under low ambient temperatures (Irving et al., 1957). Presumably, linoleic acids might have been overly abundant for our experimental animals because we aimed at enriching bats with the ^{13}C marked substance during the pre-hibernating period. Possibly, hibernators may switch to saturated fatty acids as the preferred oxidative fuel when unsaturated fatty acids become scarce in adipocytes. Indeed, it would be interesting to enrich animals with varying amounts of ^{13}C-marked linoleic acid and compare differences in APE values between torpor and arousal events, and between early and late hibernation. Consistent with Ruf and Arnold (2008) and Ben-Hamo and colleagues (Ben-Hamo et al., 2011), we observed a decrease in body temperature in animals fed linoleic acid compared with conspecifics fed palmitic acid. Again, varying the amount of fed linoleic acid in future experiments might shed light on the exact relationship between the abundance of linoleic acid in hibernators and the relative decrease in body temperature. In our experiment, we only used live animals because of the conservation status of the experimental species. Thus, we cannot reveal the level of ^{13}C enrichment in triacylglycerols of adipocytes and in cell membranes, particularly in membranes of cardiomyocytes.

Conclusions

Our approach of marking triacylglycerol in adipocytes with ^{13}C-enriched linoleic acid or palmitic acid revealed new insights into the selective use of fatty acids as oxidative fuels in a mammalian hibernator (see also Welch et al., 2015; McCue and Welch, 2016). Monitoring the breath of ^{13}C-marked hibernators in real time with laser spectroscopes enabled us to estimate the selective use of fatty acids as an oxidative fuel for hibernation. We found no support for a prudent use of PUFA in our study animals, and no change in the relative use of ^{13}C-enriched fatty acids during the course of hibernation, which might be explained by faster transportation rates of PUFA compared with SFA, and also the fact that our animals might not have been short of linoleic acid during the hibernation period.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.V.; Methodology: C.V.; Validation: C.V.; Formal analysis: E.R.; Investigation: E.R., C.V.; Resources: C.V.; Data curation: C.V.; Writing - original draft: E.R., C.V.; Writing - review & editing: E.R., C.V.; Visualization: C.V.; Supervision: C.V.; Project administration: C.V.; Funding acquisition: C.V.

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Data availability
Data are available from the Dryad digital repository (Weise and Voigt, 2017): https://doi.org/10.5061/dryad.hr744.

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