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Source: Journal of Vertebrate Biology, 72(23036)

Published By: Institute of Vertebrate Biology, Czech Academy of Sciences

URL: https://doi.org/10.25225/jvb.23036

Journal of Vertebrate Biology

Open Access

J. Vertebr. Biol. 2023, 72: 23036 DOI: 10.25225/jvb.23036

RESEARCH PAPER

Hibernation strategy – related profound differences in the whole-body fat composition of bats

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Received 20 March 2023; Accepted 16 June 2023; Published online 10 August 2023

Abstract. Bats can use a wide range of roosts as hibernacula, resulting in diverse hibernation strategies. The ecological needs of a species during hibernation translate into particular torpor-arousal patterns and physiological demands. For mammalian hibernators, the oxidation of fatty acids from triacylglycerols stored in white and brown adipocytes provides the main energy to fuel hibernation. The relative content of saturated, monounsaturated, and polyunsaturated fatty acids in body fat brings multifarious costs and benefits, and their importance during hibernation is likely changing. While considering the level of fatty acid saturation and their properties, we hypothesised that whole-body fat composition varies between bat species (*Nyctalus noctula*, *Myotis myotis*) that employ different hibernation strategies. Therefore, the focus of this study was to determine the relative fatty acid composition of the whole-body fat of these species. We found evidence that the body fat of *N. noctula* has a higher relative content of MUFAs than *M. myotis*, which, on the other hand, has high SFAs and PUFAs. Such profound differences in fatty acid profiles suggest that the studied species' distinct hibernation strategies and torpor-arousal patterns are reflected in functional differences.

Key words: energy reserves, fatty acids, PUFA, MUFA, SFA

Introduction

Torpor and hibernation represent powerful strategies enabling animals to cope with periods of low food availability and unsuitable environmental conditions (e.g. short photoperiod or challenging weather). For seasonal hibernators, entrance into hibernation is anticipated several weeks in advance by changes in behaviour and physiology that lead to the accumulation of energy stores (Kunz et al. 1998, Speakman & Rowland 1999). These changes are reflected in an adjustment of the thermoneutral zone and a decrease in basal metabolic rate (BMR) (Ruf & Geiser 2015). During hibernation, individuals achieve a minimum torpid metabolic rate of 4% of BMR and a variable reduction of their body temperature (T_b), ranging on average for most species between 0 and 10 °C (Ruf & Geiser 2015). Hibernation corresponds to multiple and successive torpor bouts lasting for days to a few weeks, during which animals rely entirely on fuel stores, such as body fat and/or food caches (Humphries et al. 2003, Dark 2005). Torpor bouts are periodically interrupted by brief arousals to a euthermic state, which consumes up to 85% of winter energy stores (Thomas et al. 1990). As torpor entails several potential costs (Humphries et al. 2003,

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Boyles et al. 2020), torpor expression and quantitative energetics are influenced by species, environmental conditions, and individual states, including available energy reserves (Boyles et al. 2007, Czenze et al. 2017, Blažek et al. 2019, Bachorec et al. 2021, E. Bachorec, unpublished data). The main energy source during periods of food scarcity is stored in the white and brown adipose tissue as triacylglycerols (TGA). In addition to an increased mass of both types of adipose tissue (Feist et al. 1986, Kunz et al. 1998, McGuire et al. 2009), heterothermic mammals also increase the proportion of polyunsaturated fatty acids (PUFA) in their body fats prior to entering hibernation (Schalk & Brigham 1995). The seasonal increase in PUFAs, which have a characteristic of low melting points, is important in maintaining the fluidity of depot fats for fuelling metabolism during torpor, as well as the fluidity of membrane phospholipids at low body temperatures (Irving et al. 1957, Mead 1986, Aloia 1988). Besides the effect of the total amount of fat reserves on torpor expression in terms of optimal hibernation theory (Boyles et al. 2020), the fatty acid composition of body fat also influences the utilisation of torpor and metabolic rate (MR) of animals. Previous studies concluded that an increased relative proportion of PUFAs in fat reserves of hibernators resulted in decreased T_b and prolonged torpor bouts and ultimately increased survival during the hibernation season (Geiser et al. 1990, 1992, Frank 1992, Florant et al. 1993). Some evidence also indicates that monounsaturated fatty acids (MUFA) may positively affect torpor, i.e. longer torpor bouts, lower T_b and MR (Frank & Storey 1996, Geiser et al. 1994). On the other hand, depot fat, rich in saturated fatty acids (SFA), increases metabolic rate and T_b, which may explain differences in MR among torpid animals (Geiser 1993). Additionally, fatty acids are not uniformly mobilised from adipocytes; some are preferentially mobilised while others are preferentially retained (Raclot & Groscolas 1993, Price et al. 2013). Several studies have observed that short-chain SFAs and fatty acids with one or more double bonds are mobilised and metabolised quicker in fish, birds, and mammals (Raclot & Groscolas 1995, Sidell et al. 1995, Price et al. 2008, 2013). For mammalian hibernators, the synthesis of MUFAs and the specific retention of PUFAs acquired through feeding might be the only way to increase the proportion of unsaturated fatty acids in their fat depot. Insectivorous bats, which have access to only low levels of PUFA, should select their insect prey to maximise PUFA intake (Schalk & Brigham 1995). Voigt et al. (2019) argue that the diet of most insectivorous bats is low in PUFA content. Thus consumption of a diet enriched in MUFA and synthesis of MUFA are mechanisms to incorporate unsaturated fatty acids in depot fat, as suggested for hibernating echidnas (Falkenstein et al. 2001).

Hibernation, however, can be employed in various forms. Bats can use a wide range of natural and artificial structures as roosts. While caves provide a stable thermal microclimate for hibernating bats such as greater mouse-eared bats *Myotis myotis* (Zukal et al. 2005, 2017), tree roosts and crevices in buildings used by common noctule bats *Nyctalus noctula* provide much less insulation from the external daily T_a cycle (Sluiter et al. 1973, Turbill 2006). *Nyctalus noctula* have been observed to hibernate in road bridge crevices at T_a of –13 °C (Ceľuch & Ševčík 2008). Situations like this often force bats to arouse from torpor and move to a proper shelter. As such, noctules are well adapted for hibernation in harsh microclimatic conditions. Additionally, in contrast to *M. myotis*, *N. noctula* is noticeable by flight and foraging activity during winter (also at $T_a < 0$ °C) (Gaisler et al. 1979, Avery 1986, Ceľuch & Kaňuch 2005, Kaňuch et al. 2005). Therefore, comparing the relative body fat composition of two species with different ecology and roost requirements seems to be a good model for testing proximate mechanisms of thermal response and hibernation patterns. We hypothesise that the two species will differ in the whole-body fatty acid composition. Regarding the character of hibernation strategy, we predict that *N. noctula* would benefit from high content of easily and rapidly metabolizable fatty acids (MUFA, PUFA), while *M. myotis* would rely on energy-dense fatty acids (SFA).

Material and Methods

Sample preparation and analysis

Whole body fatty acid composition was compared across 18 common noctule bats and 14 greater mouse-eared bats (Table S1). All bats died during the second part of deep hibernation season (late February-mid March). *Nyctalus noctula* cadavers were collected within a few days after disposing of them from their shelter in a public building. *Myotis myotis* cadavers were collected within a 3-week period during regular data collection visits (Bachorec et al. 2021). Before that, bats were naturally hibernating and fasting. As we only received the cadavers, we could not influence the sampling design and used the material available.

Sample preparation and analysis took place during the summer of 2022. Cadavers without heads and wings were homogenised, and 16 g samples were prepared from each. Total fat content was determined gravimetrically, where samples (3-5 g) were acid

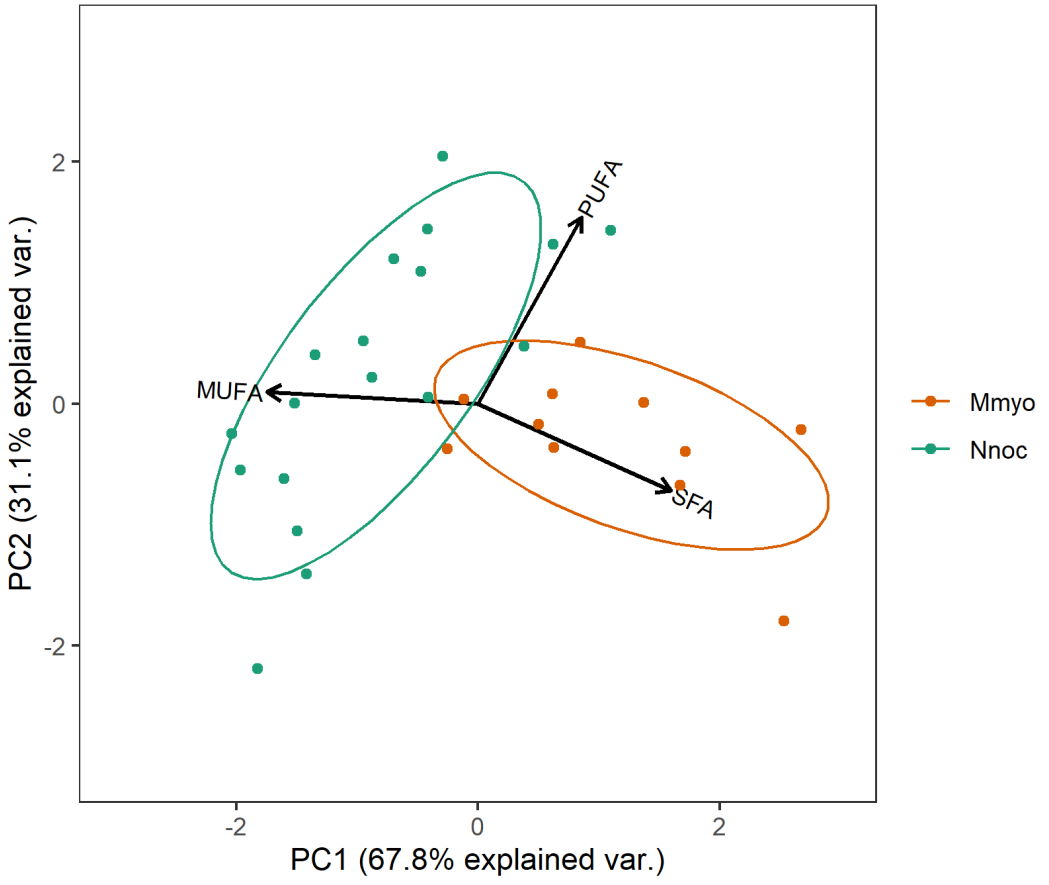

Fig. 1. Principal component analysis based on the whole-body fatty acid proportions of two bat species grouped by the level of saturation.

hydrolysed via 50 ml of boiling diluted hydrochloric acid (4 mol/l) for lipids to be released. Afterwards, the hydrolysed sample was filtered and dried for 1 hour at 103 °C. The fat extraction procedure was performed using the Soxtec 2050 (FOSS, Denmark) petroleum ether extractor. For the determination of fatty acids, extracted fat samples were esterified to fatty acid methylesters via transmethylation using methanolic solution of potassium hydroxide (2 mol/l). Extracted fatty acid methylesters were separated, identified, and quantified using high-performance capillary gas chromatography with FID detector (HP5890A, Germany). The fat and fatty acid analyses were performed by an external company (State Veterinary Institute, Czech Republic) following ISO standards (ISO-1443; ISO-12966-2; ISO-12966-4).

Statistical analyses

Fatty acids that showed no variability and/or had a low proportion relative to the total fat profile were excluded from the comparison. These included caproic (0.01%), capric (0.02%), caprylic (0.01%), nervonic (0.04%), and undecanoic acid (0.01%). Principal components analysis (PCA) via '*prcomp*' function was employed to summarise the variation and detect distinction in the fatty acid profiles of whole-body fat between *N. noctula* and *M. myotis*. PCA was performed on all fatty acids pooled into three categories based on the saturation level (SFA, MUFA, PUFA) and the specific fatty acids in these categories alone. As our data are in the form of proportions relative to the total fatty acid profile, a non-parametric test was used to test the differences between the two species. Therefore, we used npMANOVA using the '*adonis2*' function from the '*vegan*' package (Oksanen et al. 2013), which is a permutational analysis using distance matrices and is appropriate for comparing relative proportions. To assess differences between species, a comparison was carried out on all fatty acids pooled together and with fatty acids grouped into categories based on the saturation level. Analyses were performed in R Studio (R Core Team 2022).

Results

Oleic acid had the highest proportion to the total fatty acid profile, ranging from 49.7-71.3% in *N. noctula* and 32.4-59.8% in *M. myotis*. On average, a high proportion was also observed in linoleic acid (13.9 and 12.6%), palmitic acid (9.4 and 12.7%) and stearic acid (4.3 and 9.4%) in *N. noctula* and *M. myotis*, respectively. The mean values of the relative fatty

Table 1. Relative fatty acid content (mean % to total fat) and PCA scores for fatty acids grouped by the level of saturation. $N_{N.noct}$ = 18; $N_{M.myo}$ = 14.

	FA		$\bar{X}$ *N. noctula* n = 18	$\bar{X}$ *M. myotis* n = 14	PC1	PC2
SFA	lauric	C12.0	0.112	0.074	–0.258	–0.490
	tridecanoic	C13.0	0.124	0.024	–0.267	–0.321
	myristic	C14.0	0.919	0.799	–0.095	–0.673
	pentadecanoic	C15.0	0.054	0.236	0.197	0.213
	palmitic	C16.0	9.419	12.744	0.249	–0.001
	heptadecanoic	C17.0	0.132	0.696	0.357	–0.084
	stearic	C18.0	4.294	9.440	0.328	–0.050
	arachidic	C20.0	0.247	1.176	0.351	–0.199
	heneicosanoic	C21.0	0.013	0.354	0.332	–0.198
	behenic	C22.0	0.056	0.776	0.341	–0.197
	tricosanoic	C23.0	0.927	2.656	0.281	–0.085
	lignoceric	C24.0	0.160	1.502	0.297	–0.152
MUFA	myristoleic	C14.1_5	0.167	1.872	–0.043	0.774
	palmitoleic	C16.1_7	4.937	2.176	0.395	0.479
	cis-10-heptadecenoic	cC17.1_9	0.111	0.201	–0.162	–0.097
	oleic	C18.1_9	61.942	46.658	0.486	–0.155
	elaidic	tC18.1_9	0.083	0.224	–0.407	0.225
	gondoic	C20.1_9	0.345	1.441	–0.526	0.175
	erucic	C22.1_9	0.044	0.295	–0.369	–0.236
PUFA	α-linolenic	C18.3_3	0.968	0.991	0.295	0.013
	γ-linolenic	C18.3_6	0.023	0.214	0.308	–0.030
	linoleic	C18.2_6	13.881	12.262	–0.035	0.157
	linolelaidic	tC18.2_6	0.054	0.117	0.321	–0.405
	cis-11,14,17-eicosatrienoic	cC20.3_3	0.018	0.064	0.350	0.389
	cis-11,14-eicosadienoic	cC20.2_6	0.131	0.783	0.315	0.464
	dihomo-γ-linolenic	C20.3_6	0.071	0.143	0.293	0.463
	arachidonic	C20.4_6	0.013	0.216	0.341	–0.324
	EPA	C20.5_3	0.101	0.154	0.312	–0.049
	cis-13,16-docosadienoic	C22.2_6	0.012	0.061	0.315	–0.229
	DHA	C22.6_3	0.556	0.944	0.304	–0.263
pooled	SFA		16.496	31.017	0.634	–0.423
	MUFA		67.664	51.245	–0.694	0.056
	PUFA		14.973	17.731	0.340	0.904

acid content and PCA scores are shown in Table 1. The PCA based on fatty acid composition showed separation of two species regarding the proportion of SFA and MUFA with PC1 and PC2 explaining 98.9% of variability (Fig. 1). Clear separation of two species where with PC1 and PC1 explained 68.3% of variability was also observed in proportions of the specific SFAs, with the short chain SFAs (C12-C14) representing *N. noctula* and the long chain SFAs (C15-C24) representing *M. myotis* (Fig. S1). The two bat species were clearly separated regarding MUFAs, with 71.8 % variability explained by the first two PCs (Fig. S2). A positive correlation of oleic and palmitoleic acid with PC1 characterises *N. noctula*, while the negative correlation of elaidic, erucic, gondoic and cis-10-heptadecenoic acid represents *M. myotis*. The principal component analysis of PUFAs composition, which explained 61% of variability on PC1 and PC2,

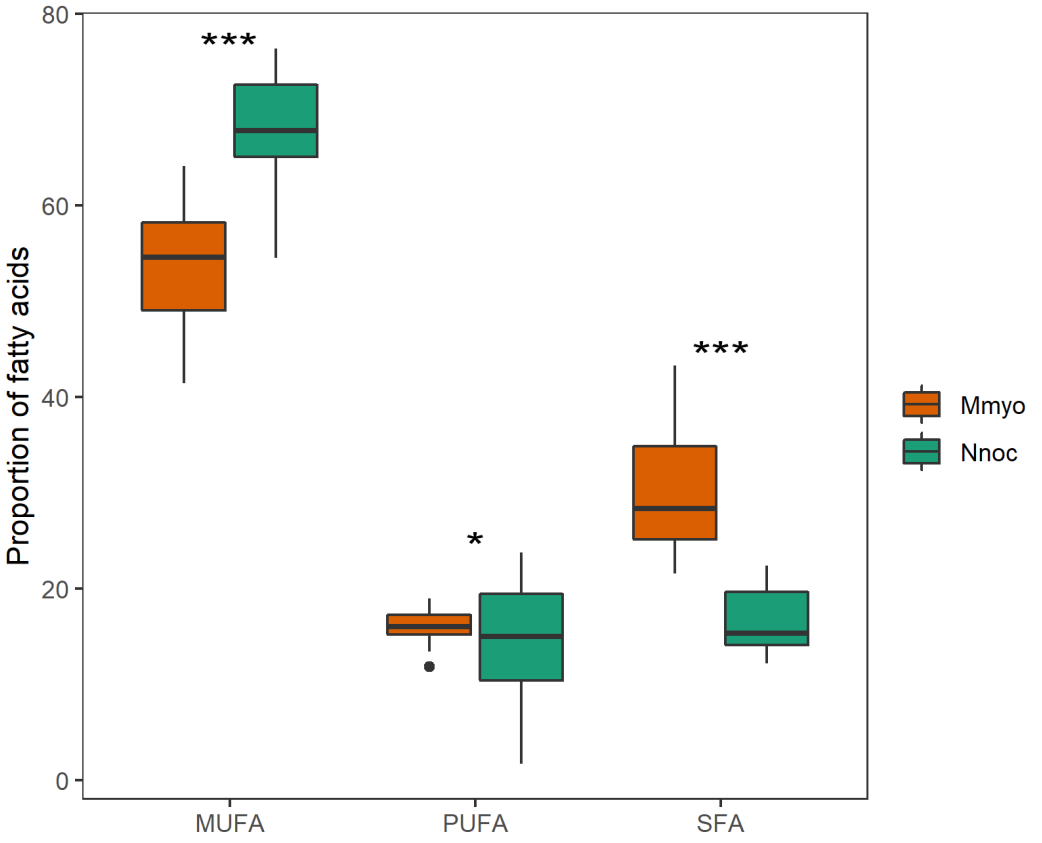

Fig. 2. Proportions of the whole-body fatty acids as percentage relative to the total fat grouped into categories by the level of saturation.

shows a positive correlation of all PUFA types with *M. myotis* and practically all *N. noctula* are centralised within negative PC values (Fig. S3). All PCA based on fatty acid composition explained more than 60.0% of the total variability.

The npMANOVA supported these distinctions by providing evidence of profound differences in the whole-body fatty acid composition between the two bat species under study. Species showed a significant effect on the fatty acid composition when proportions of all fatty acids were pooled ($F_{1,28}$ = 19.416; partial R_2 = 0.409; $P < 0.001$). When fatty acids were divided into categories (SFA, MUFA, PUFA, Fig. 2), the npMANOVA showed strong evidence that the proportion of SFAs ($F_{1,28}$ = 20.567; partial R_2 = 0.423; $P < 0.001$) was higher in *M. myotis* and that the proportion of MUFAs ($F_{1,28}$ = 23.549; partial R_2 = 0.456; $P < 0.001$) was higher in *N. noctula*. This difference is present, however weaker, also in the case of PUFAs ($F_{1,28}$ = 4.76; partial R_2 = 0.145; $P < 0.05$), with *M. myotis* showing a slightly increased proportion of PUFAs.

Discussion

We compared the whole-body fatty acid composition between two temperate bat species that use different hibernation strategies. Consistent with our prediction, the results show profound differences in the fatty acid composition of these two species. The fatty acid composition of body fat has been shown to reflect that of diet (Florant 1998, Falkenstein et al. 2001), although processes such as post-absorptive modification and *de-novo* synthesis cannot be ignored (Price 2010). It is known that *N. noctula* feeds mainly on Diptera, which are low in PUFAs, although Coleoptera and Lepidoptera are also important components of its diet (Schalk & Brigham 1995, Vaughan 1997), while *M. myotis* feeds primarily on large Coleoptera species (mainly Carabidae and Scarabaeidae) richer in PUFA (Arlettaz & Perrin 1995, Beck 1995, Schalk & Brigham 1995, Andreas 2002). Foraging habits and prey selection, especially during the pre-hibernation season, might result in divergent body fat composition to meet the specific needs of animals during hibernation (Schalk & Brigham 1995, Kunz et al. 1998).

Differences demonstrated by our results raise questions about differential energy availability and utilisation rate between the studied species during winter. Analysis of the whole-body fatty acid composition revealed that the proportion of SFAs to the total body fat was higher in *M. myotis*. For hibernating animals, SFAs present the main

energy source (Florant et al. 1990, Geiser 1991, Price et al. 2013). Long-chain saturated fatty acids are energetically dense. However, they are mobilised and metabolised slower than unsaturated and shorter fatty acids (Price & Gugliemo 2009, Price 2010). The difference in SFA content likely reflects the energetic needs for a different length of the hibernation period which is shorter for *N. noctula* (mid-December-March) (Ceľuch & Kaňuch 2005, Zahn & Kriner 2016) compared to *M. myotis* (mid-November-mid-April) (Zukal et al. 2005, 2017). The higher content of energetically dense saturates in the body fat of *M. myotis*, conspicuous by deep, prolonged hibernation (Zukal et al. 2017), might thus represent a long-term energy store to fuel the overwinter demands. However, because body fats must remain fluid to be metabolised at low T_b, some level of unsaturation is needed, which is attained via specific retention of fatty acids with one or more double bonds (Irving et al. 1957, Mead 1986, Aloia 1988). We observed a high proportion of MUFAs (especially oleic acid) in both species. A high proportion of oleic acid in *N. noctula* was also observed by Voight et al. (2019), and the same was reported for several other bat species (Ewing et al. 1970, Levin et al. 2013, McGuire et al. 2013) as well as echidnas (*Tachyglossus aculeatus*) by Falkenstein et al. (2001). As one double bond has the most profound effect on fatty acid fluidity (Cossins & Lee 1985), it appears that high content of MUFAs can compensate for low dietary PUFA. It is the most likely way to cope for insectivores, which typically have a low PUFA content in their diet compared to herbivorous hibernators (Falkenstein et al. 2001). Moreover, high PUFA and MUFA content in body fat has positive effects on torpor expression (Geiser et al. 1990, 1992, 1994, Frank 1992, Florant et al. 1993, Frank & Storey 1996, Falkenstein et al. 2001) and thus improve energy conservation. Evidence of higher MUFA content at the expense of SFAs revealed by PCA and supported by MANOVA possibly accounts for the specific needs of *N. noctula*. This species hibernates in harsh, unstable conditions that may compel them to arouse and fall into torpor more frequently and quickly (Sluiter et al. 1973, Ceľuch & Kaňuch 2005, Kaňuch et al. 2005). Selective mobilisation of fatty acids could notably affect the supply of tissues and organs with specific fatty acids in situations of negative energy balance (Raclot & Groscolas 1993). We suggest that a higher content of MUFAs provides a rapid energy source for thermogenesis during short and mild arousals of *N. noctula*. Furthermore, hibernation shelters used by *N. noctula* often allow them to benefit from passive rewarming. By hibernating in a roost exposed to the daily T_a cycle, they can rewarm more effectively and save considerable energy during arousals that occur during the increased T_a (Turbill & Geiser 2008). On the other hand, the slightly higher content of PUFAs in *M. myotis* is likely to fuel body tissues during long torpor bouts. Although BMR does not differ much between the studied species, *N. noctula* has a higher Q_{10} (3.4) compared to *M. myotis* (3.0) (Geiser 2004). This difference suggests that metabolic processes, including warming up during arousal, should be faster than in *M. myotis*. As reported by Rosner & Voight (2018), *N. noctula* rewarms in 40 min, with a peak MR after 25-28 min, faster than *M. myotis*, which rewarms after 60 min with peak MR at 35 min (E. Bachorec, unpublished data). However, a bigger differential between the species might be expected.

Altogether, a high MUFA content relative to the total fatty acid profile appears to be a general trait of hibernating insectivores. It provides hibernators with a constitution of fat that has suitable physical properties for deep torpor and periodic arousals. However, as our results showed, the fatty acid profile might vary with different hibernation strategies. The endogenous synthesis of MUFAs is independent of diet and seems to be an efficient adaptive mechanism that provides hibernators with a low PUFA diet to survive the dramatic changes in physiology during overwintering without additional fat restructuration. Even though our sampling was not standardised and might have accounted for some variance, the results presented in this study contribute to the knowledge about the fatty acid composition of hibernating bats and provide more detailed insight and improvement in modelling energetic costs during hibernation.

Acknowledgements

We are very grateful to Tomáš Bartonička for providing the cadavers of Nyctalus noctula *for the study and to Tomáš Heger for his help during the fieldwork. We would also like to thank Martin Kuba (State Veterinary Institute, Czech Republic) for his help with the determination of fat composition. This study was partially supported by Project IGA 216/2021/FVHE.*

Author Contributions

E. Bachorec, J. Pikula and J. Zukal conceived and designed the study; J. Zukal and K. Zukalová collected material, with support from J. Pikula; K. Zukalová and V. Seidlová performed the laboratory analyses; E. Bachorec analysed the data; E. Bachorec and J. Zukal drafted the manuscript, to which all authors contributed with critical comments.

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Supplementary online material

Table S1. Dataset supporting the study's findings (proportions of fatty acids relative to whole body fat) (https://www.ivb.cz/wp-content/uploads/JVB-vol.-72-2023-Bachorec-et-al.-Table-S1.xlsx).

Principal component analysis based on the whole-body fatty acid proportions of two bat species: **Fig. S1**. Saturated fatty acids, **Fig. S2.** Monounsaturated fatty acids, **Fig. S3.** Polyunsaturated fatty acids (https://www.ivb.cz/wp-content/uploads/JVB-vol.-72-2023-Bachorec-et-al.-Fig.-S1-S2-S3.pdf).