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RESEARCH ARTICLE

Stable carbon isotopes in breath reveal fast metabolic incorporation rates and seasonally variable but rapid fat turnover in the common shrew (*Sorex araneus*)

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ABSTRACT

Small non-migratory mammals with Northern distribution ranges apply a variety of behavioural and physiological wintering strategies. A rare energy-saving strategy is Dehnel's phenomenon, involving a reduction and later regrowth of the body size, several organs and parts of the skeleton in red-toothed shrews (Soricidae). The size extremes coincide with major life stages. However, the physiological consequences for the shrew's metabolism remain poorly understood. In keeping with the energetic limitations that may induce the size changes, we hypothesised that metabolic incorporation rates should remain the same across the shrews' lifetimes. In contrast, fat turnover rates should be faster in smaller subadults than in large juveniles and regrown adults, as the metabolic activity of fat tissue increases in winter individuals (subadults). Measuring the changes in the ratio of exhaled stable carbon isotopes, we found that the baseline diet of shrews changed across the season. A diet switch experiment showed that incorporation rates were consistently rapid (t_{50}=38.2±21.1–69.3±53.5 min) and did not change between seasons. As predicted, fat turnover rates were faster in size-reduced subadults (t_{50}=2.1±1.3 h) compared with larger juveniles (t_{50}=5.5±1.7 h) and regrown adults (t_{50}=5.0±4.4 h). In all three age/size classes, all body fat was turned over after 9–24 h. These results show that high levels of nutrient uptake are independent of body size, whereas fat turnover rates are negatively correlated with body size. Thus, the shrews might be under higher pressure to save energy in winter and this may have supported the evolution of Dehnel's phenomenon.

KEY WORDS: Metabolism, Diet switch experiment, Wintering adaptation, Size change, Dehnel's phenomenon

INTRODUCTION

Harsh winter conditions can be challenging for small non-migratory mammals with Northern distribution ranges. A variety of strategies to survive these conditions exist, including hibernation, food caching and overall reduced activity (Heldmaier et al., 2004; McNab, 2010; Taylor et al., 2013). Animals that remain active all year need particularly effective foraging strategies (Churchfield, 2002), efficient food-incorporation mechanisms to fuel their metabolism (Hobson and Stirling, 1997; Tieszen et al., 1983; Voigt et al., 2010) and adaptations to reduce energy expenditure, such as insulating fur (Scholander et al., 1950) or larger fat deposits (Heldmaier et al., 1985).

In contrast to these more commonly known wintering adaptations, red-toothed shrews (Soricidae) exhibit a rather unusual strategy. Some of these species reduce their small body size and mass even more in winter (Ochocinska and Taylor, 2003), in direct contradiction to 'Bergmann's rule' (McNab, 1971), which states that smaller body size results in a disadvantageous volume: surface ratio, which is detrimental to preventing heat loss. 'Dehnel's phenomenon' (Dehnel, 1949; Pucek, 1955) describes a seasonal size reduction of the body mass, parts of the skeleton, including the vertebral column length and skull (Crowcroft and Ingles, 1959; Hyvarinen, 1969; Pucek, 1963), and several major organs, including the brain (Churchfield, 1990; Mezhzherin, 1964; Pucek, 1970). Young shrews are born in early summer, quickly reach a first size peak and begin to decrease in anticipation of the cold season in late summer. After reaching a minimum size in February they begin to regrow, reaching a second size peak that coincides with sexual maturation in April/May, shortly before the end of their short lives (Churchfield, 1990). In the best known example, the common shrew (*Sorex araneus*), body mass varies between 8–9 g (summer juveniles), 6 g (size-reduced winter subadults) and 11–12 g (regrown spring adults) (Churchfield, 2002; Hanski, 1984; Ochocinska and Taylor, 2003). Their less favourable body-surface:mass ratio in winter forces *S. araneus* to invest large parts of their absolute energy expenditure into heat production (Taylor et al., 2013), which is partly compensated by improved fur insulation (Borowski, 1959; Ivanter, 1994). Recently, Dehnel's phenomenon has also been described in weasels, which share several important life history traits with red-toothed shrews (Dechmann et al., 2017). This supports the general hypothesis that energy savings and/or a reduction in resource requirements are driving forces behind the evolution of this phenomenon, but the consequences for the animals are still poorly understood (Dechmann et al., 2017; LaPoint et al., 2017). In summary, Dehnel's phenomenon is thought to be an energy-saving strategy, allowing the shrews to reduce absolute food requirements when the prey abundance is lower in the cold season (Churchfield, 2002; Gliwicz and Taylor, 2002; Hyvarinen, 1969; McNab, 1991; Pucek, 1970; Taylor et al., 2013) and/or energetic costs through the reduction of high energy-consuming tissues, including the brain.

Typical for soricid shrews, *S. araneus* have an exceptionally high metabolic rate and short fasting endurance, and are unable to use torpor, resulting in a constant need for food (Churchfield et al., 2012). They eat more than their own body mass of food per day, depending on diet quality (Hanski, 1984; Rychlik and Jancewicz, 2002). They must then efficiently digest large quantities of food to make energy available rapidly throughout the year. *Sorex*

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araneus have many activity cycles per day, where they forage ~55 min until their stomach is full, followed by short resting or sleeping periods of ~64 min (Saarikko and Hanski, 1990). Consequently, they appear to be limited by the assimilation rates of the digestive tract (Saarikko and Hanski, 1990). Using a non-invasive method, such as measuring changes in the ratio of stable carbon isotopes in exhaled breath in a diet switch experiment, one can determine how quickly recently ingested food is incorporated into the metabolism. The time until 50% of metabolism is fuelled by ingested food (t_{50}) generally scales positively with body size (Carleton and Del Rio, 2005). In addition, diet composition can impact incorporation rates (Voigt et al., 2008a). But, although shrews change body size throughout their lifetime, their digestive tract is most likely working at constant high rates to make energy from the recently eaten food available to the organs.

In addition to incorporation rates, stable isotopes in the breath can also be used to determine fat turnover rates, i.e. the t_{50}, which is defined as the time until 50% of the carbon atoms in the stored fat have been replaced by carbon atoms from the recently ingested diet. This can be used to calculate a proxy for how long an animal can survive without food, even though other factors might also influence the starvation time of an animal, such as carbohydrate or protein metabolism (McCue, 2010). One of the fastest fat turnover rates has been found in the broad-tailed hummingbird, with a t_{50} of 0.8 days (Carleton et al., 2006). Like many small animals, the ability of *S. araneus* to deposit fat reserves is poor, which makes them even more susceptible to food shortage (Churchfield, 1981; Myrcha, 1969). Under natural conditions, *S. araneus* mainly have reserves of brown adipose tissue (BAT) (Myrcha, 1969), which is primarily used to warm blood coming from the extremities (Przełecka, 1981). This strategy of heating up blood in the body core by burning BAT and transferring the blood back to the extremities is assumed to be one explanation for the successful wintering of shrews (Hyvarinen, 1994). In fact, relative and absolute whole-body fat content is higher in winter, reaching a maximum in January, when body size and mass are at a minimum, and then decreasing until March (Myrcha, 1969). In contrast, BAT metabolic activity peaks in winter (Przełecka, 1981). In captivity, the survival time of *S. araneus* without food is 5–10 h (Gliwicz and Taylor, 2002; Hanski, 1994; Taylor, 1998), confirming that energy reserves are metabolised quickly.

After ingestion, nutrients enter metabolic processes, and the risk of starvation may be one of the physiological aspects that guide the seasonal reversible size changes in shrews. Our goal was to test whether the incorporation rate of recently digested food and the fat turnover rate changes between large summer juveniles, small winter subadults and regrown, sexually mature, spring adults. We used the amount of stable carbon isotopes in the breath of shrews to non-invasively measure metabolic incorporation and fat turnover rates in wild-caught *S. araneus*. Because the relative resting metabolic rate of shrews does not change at least between summer juveniles and winter subadults (Taylor et al., 2013), and energy uptake is limited by the digestive tract's physiology (Saarikko and Hanski, 1990), we assumed that the metabolic incorporation rate should remain constantly high throughout the year. Secondly, we hypothesised that, because overall fat stores are small and metabolic rates constantly high, fat turnover rate should be exceptionally high and result in a short t_{50} and thus a survival time of only a few hours, when other energy supplying mechanisms such as protein or carbohydrate metabolism are also taken into account. However, here we expected differences between the age classes: as the absolute and relative BAT amount increases in winter along with its metabolic activity also increasing, fat turnover rate should increase and fat stores should be replaced faster from recently ingested food in winter subadults compared with summer juveniles and spring adults.

We collected breath samples in a diet switch experiment and recorded the amount of consumed food. This combination of morphological and physiological data gives novel insight into how shrews use resources and convert them into energy throughout the seasons. As we collected parameters indicative of Dehnel's phenomenon, such as body mass and skull height standardised by length of the tooth row, we were able to put the data collected in the diet switch experiment in context of this phenomenon. This will contribute importantly to the understanding of the driving forces behind the evolution of Dehnel's phenomenon in free-ranging animals, and with that of a poorly known but fascinating alternative wintering strategy in small high-metabolic mammals with a Palearctic distribution.

MATERIALS AND METHODS

Trapping of animals

All handling and sampling methods were approved by the Regierungspräsidium Freiburg, Baden-Württemberg (35-9185.81/G-15/128). We trapped a total of 29 shrews (*Sorex araneus* Linnaeus 1758) between 07:00 and 11:00 h in the forest and meadows in Möggingen, Southern Germany (longitude 8.994, latitude 47.766) in June (large juveniles, N=12), January/February (size-decreased subadults, N=9) and April/May (regrown adults, N=8+1 recapture from January) 2016 with wooden live-traps (Jerzy Chilecki, Białowieża, Poland) baited with mealworms and checked at 2-h intervals.

Age determination and housing of animals

We classified individuals as juvenile, subadult or adult based on the annual life cycle of the shrews and external morphological characteristics (Dehnel, 1949; Pearson, 1945; Pucek, 1955). We transferred suitable animals (only adults are sexually mature; pregnant females were excluded) to a holding cage immediately without food until the onset of the experiment, a maximum of 1 h after capture. During the 3–5 days of the experiment, each shrew was housed individually under ambient conditions in two connected cages with deep soil bedding, enriched with nesting material, and a running wheel. Water was provided *ad libitum*; for the feeding regime, see below.

Diet and breath collection

We assessed the rate at which *S. araneus* incorporate recently digested food into their metabolism (incorporation rate) and the rate at which the animals turn over their body fat in a diet switch experiment by measuring the stable carbon isotope ratios of the exhaled breath. Shrews were not fed between capture and the collection of the first sample to ensure that their exhaled breath reflects the stable carbon isotope ratios of their natural diet. We assumed that their natural diet contained mainly insects and earthworms that feed on C3 plants (Churchfield, 2002; Churchfield et al., 2012; Gliwicz and Taylor, 2002). We then fed shrews a mixture of chicken and corn-fed crickets (*Gryllus assimilis*). We collected samples of this ^{13}C-enriched C4-labelled diet from each prepared batch for stable carbon isotope analysis and dried them at 40°C in a drying oven. The samples were combusted and analysed with a Flash elemental analyser 1112 (Thermo Scientific, Bremen, Germany) coupled to a Delta-Advantage isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) with a precision always better than ±0.1‰ (1 s.d.). Stable carbon isotope ratios [$\delta^{13}C_{V\text{-}PDB}$; abbreviated as $\delta^{13}C$ (a measure of the ratio of stable isotopes

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^{13}C:^{12}C)] are reported in the delta notation in parts per mil, or mUrey deviation from the international standard Vienna-PeeDeeBelemnite. Analysis showed that the chicken/cricket diet ($\delta^{13}C_{diet}$=−17.1 ±0.7‰) clearly differed from the natural diet in isotopic signature ($\delta^{13}C_{start}$=−27.2±0.5‰ to −28.9±0.7‰).

We took the shrews' masses before and after each sample collection with a digital scale (Sartorius U5000D, Göttingen, Germany; ±0.01 g). For the collection of each breath sample, we placed shrews in a sealed plastic box (550 ml volume) or plastic bottle (500 ml volume). The shrews were able to move freely in the sampling container and we monitored the activity of the shrews during the experiment visually. Ambient air was scrubbed of CO_2 using NaOH droplets in a wash bottle, connected to an aquarium pump via silicon tubing. The washed air was flushed through a plastic tube connected to the plastic box containing the shrew with a flow rate of 50 l h^{-1}. Prior to sample collection, the pump was switched off and we allowed the shrew's breath to accumulate for 2 min. We then collected the exhaled breath via a 0.90 mm diameter (20 G) hypodermic needle that was silicon-sealed into the wall of the container. Breath was collected in exetainers (Labco, Buckinghamshire, UK) by piercing the Teflon membrane with the needle attached to the sealed container. Shrews have a daily CO_2 production of approximately 2.07 l CO_2 day^{-1} (Ochocińska and Taylor, 2005), which is 1.44 ml CO_2 min^{-1}. We therefore expected the shrew's breath CO_2 to accumulate to ∼0.58% in 2 min. After each sample collection, the pump was turned back on and the air in the bottle was flushed with CO_2-free air for a minimum of 5 min. We took a baseline sample of each unfed shrew a maximum of 1 h after removing the shrew from the trap (sample '0 min'). The animal was then given approximately 2 g of the C4 diet while it remained in the experimental container and the amount of consumed food was recorded.

Incorporation rate in three different age/size classes of *S. araneus*

We collected samples from large juveniles, size-decreased winter subadults and regrown adults at 10, 20, 40, 60 and 90 min following first food uptake after the '0 min' baseline sample to measure incorporation rates. The shrew was then placed in a housing cage equipped with a running wheel and was food deprived during the next 90 min until the next sample was collected (see below) but given *ad libitum* access to water.

Fat turnover rate in three different age/size classes of *S. araneus*

To estimate fat turnover rate, we then collected samples at 3, 6, 9, 24, 48, 72 and 96 h after the '0 min' baseline sample from juveniles and adults. We collected only until the 48 h sample from subadults. After every sample collection, the shrew was returned to its home cage, where it immediately received 2 g of food and had access to a running wheel. We continued to feed each shrew its own body mass of food per day throughout the duration of the experiment (approximately 8–9 g for juveniles, 6 g for subadults and 12 g for adults) and recorded the amount of consumed food between sampling events. Before collecting each breath sample, we deprived the shrews of food for approximately 90 min to ensure that they used only endogenous substrate to fuel their metabolism.

Stable carbon isotope analysis

The breath samples were shipped to the Stable Isotope Laboratory of the Leibniz-Institute for Zoo and Wildlife Research, Berlin (Germany) and analysed with a GasBench II (Thermo Scientific, Bremen, Germany) connected to an isotope ratio mass spectrometer (Delta V Advantage, Thermo Scientific, Bremen, Germany). The breath samples were automatically flushed from the vacutainers in a stream of chemically pure helium. Then a gas chromatograph separated the CO_2 gas from the other gases before admitting it into the mass spectrometer in a continuous flow. We previously calibrated laboratory standard gas with the international ^{13}C reference material NBS 19 and analysed it together with the samples.

Precision was always better than ±0.1‰ (1 s.d.).

Exponential regression model

We expected changes in isotopic composition during the feeding experiment to follow an exponential regression model (e.g. Voigt and Speakman, 2007) and used a one-compartment model to estimate the fractional incorporation rate according to Carleton and Del Rio (2005):

$$\delta^{13}C_{breath}(t) = \delta^{13}C_{breath\infty} + [\delta^{13}C_{start} - \delta^{13}C_{breath\infty}] \times e^{-t\times c}, \quad (1)$$

where $\delta^{13}C_{breath}(t)$ is the stable carbon isotope ratio of the exhaled CO_2 at time t (in ‰), $\delta^{13}C_{breath\infty}$ is the asymptotic stable carbon isotope ratio of exhaled CO_2 when animals were equilibrated to the stable carbon isotope signature of the diet (in ‰), $\delta^{13}C_{start}$ is the stable carbon isotope ratio of the exhaled CO_2 at time 0 (in ‰) and c (in h^{-1} or min^{-1}) is the fractional rate of isotopic incorporation into the metabolism. For comparisons, we calculated for each individual shrew the t_{50} (in min or h), which is the time at which 50% of the carbon atoms in the metabolism have been exchanged:

$$t_{50} = -\frac{\ln(0.5)}{c}. \quad (2)$$

For the incorporation rate experiment, we fitted to each individual dataset non-linear least-squares (nls) regression models in R (version 3.2.5) based on one-pool dynamics using the 0, 10, 20, 40, 60 and 90 min and 3 h samples for each shrew. Including the 3 h sample allowed us to more accurately estimate the time point when the shrew's breath would be equilibrated isotopically to the C4 diet and reach a plateau ($\delta^{13}C_{breath\infty}$).

For the fat-turnover-rate experiment, we used the 0, 3, 6, 9, 24, 48, 72 and 96 h samples to fit the model to each adult and juvenile shrew, and the 0, 3, 6, 9, 24 and 48 h samples for subadult shrews. For each individual we fit one nls model and calculated the means and the standard deviations for each time point and age/size class.

To test for differences among the three age/size classes, we calculated mean values of parameters from the exponential regression model, including start value $\delta^{13}C_{start}$ and t_{50} value, and standardised skull height from the x-ray measurements (below) and the capture mass. We used a Kruskal–Wallis one-way ANOVA and the *post hoc* tests after Nemenyi in the PMCMR package (https://cran.r-project.org/web/packages/PMCMR/index.html) to analyse each class for significant differences and to test for differences between the asymptotic value, $\delta^{13}C_{breath\infty}$, and the stable carbon isotope ratio of the diet, $\delta^{13}C_{diet}$. To investigate the impact of body mass change (Δ body mass) during the experiment on metabolism and the change in $\delta^{13}C_{breath}$ of the shrews (ΔC), we used a Pearson correlation test.

Morphological parameters

We weighed the individuals immediately upon capture to assess the capture body mass. After completion of the experiments, we x-rayed the shrews under anaesthesia in an induction chamber (Surgivet, Dublin, OH, USA; oxygen flow rate 1 l min^{-1}, 5% isoflurane)

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connected to a Titus System (Dräger, Lübeck, Germany). We placed anaesthetised individuals into a form-fitting foam bed to ensure standardised body position. We x-rayed animals in a Faxitron MX 20 cabinet (26 kV, 6 s using an OPG Imaging Plate, Gendex, Hatfield, PA, USA) and extracted the images with a scanner (DenOptix, Gendex). We took both ventral and lateral x-ray images of the skull. While shrews were under anaesthesia, we individually marked them with subcutaneous passive integrated transponders (UNO PICO, 7×1.5 mm, Zevenaar, The Netherlands) and released them at the place of capture after recovery. One individual from January was recaptured in May and tested again (but see Results). We recorded skull height, from the tympanic rings to the dorsal surface of the braincase in the x-ray images, gauged on the electronic x-ray files using ImageJ. All x-ray measurements were taken blind regarding capture date by a single observer to avoid bias. We size-corrected skull height by the maxillary tooth row length because this parameter does not change seasonally within an individual (J. Lázaro, D. K. N. Dechmann, S. LaPoint, M. Wikelski and M. Hertel, unpublished).

RESULTS

Natural diet of wild-caught shrews

We took a baseline sample ($\delta^{13}C_{start}$) of the exhaled breath of unfed shrews a maximum of 1 h after catching to ensure that this sample reflects the stable carbon isotope ratios of their natural diet. The mean starting value ($\delta^{13}C_{start}$) was −28.9±0.7‰ for the juveniles, −27.2±0.5‰ for subadults and −28.9±0.7‰ for adults. Adults differed from subadults (H_0=5.2, P<0.001) and juveniles from subadults (H_0=5.3, P<0.001) (Fig. S1A).

Incorporation rate in three different age/size classes of *S. araneus*

We measured the rate at which ingested food was incorporated into the metabolism by using the diet switch experiment with shrews from the three different age/size classes. All shrews were active during the whole experiment and attempting to escape from the sampling container. After the initial breath sample and after receiving the first C4 food, the exhaled breath was enriched in ^{13}C and, between the 60 min and 90 min sampling, came close to the estimated asymptotic value for all age/size classes [juveniles (n=8): −23.1±2.3‰, subadults (n=7): −18.0±2.7‰ and adults (n=8): −23.3±2.6‰; Fig. 1, Table S1). The estimated asymptote of the subadults was higher than the estimated asymptotes of juveniles (H_0=4.1, P=0.011) and adult individuals (H_0=4.0, P=0.012). The estimated asymptotic values of the shrews were lower than the $\delta^{13}C$ values of the C4 diet we fed to them ($\delta^{13}C_{diet}$=−17.0±0.7‰, juveniles: H_0=8.7, P<0.001, subadults: H_0=5.2, P=0.004, adults: H_0=8.7, P<0.001). The time required to exchange 50% of the carbon atoms in the breath CO_2 with carbon atoms from the recently ingested C4 diet (t_{50}) averaged 44.9±25.2, 69.3±53.5 and 38.2 ±21.1 min for the juveniles, subadults and adults, respectively. These values did not differ from each other (Kruskal–Wallis chi-squared=1.9, d.f.=2, P=0.38; Fig. S1B). For all three age/size classes, the $\delta^{13}C_{breath}$ enrichment followed a one-pool exponential incorporation model, with $\delta^{13}C_{breath}(t)=-23.12-5.80e^{-0.02017t}$ for the juveniles, $\delta^{13}C_{breath}(t)=-18.03-9.71e^{-0.01445t}$ for the subadults and $\delta^{13}C_{breath}(t)=-23.26-6.20e^{-0.02333t}$ for the adults.

Fat turnover rate in three different age/size classes of *S. araneus*

To measure fat turnover, we continued to feed the shrews after the incorporation rate experiments with the same C4 diet. We then sampled their breath for the following 2–4 days after removing access to food for 90 min before each test to ensure that exhaled breath contained only isotopes from metabolised body reserves. We removed one shrew (the recaptured individual 21) from the adult dataset because it had an exceptionally low rate constant of isotopic incorporation into the metabolism, resulting in a high t_{50} value (Table S2). Therefore, we expected that the low and constant rate of isotopic incorporation was caused by an error in the 9 h sampling, after which a significant drop in the stable carbon isotope ratio occurred. All shrews showed enrichment of $\delta^{13}C_{breath}$ and reached an asymptotic value between 9 and 24 h sampling of −17.8±0.3, −18.1±0.9 and −17.7±0.5‰ for juveniles (n=9), subadults (n=8) and adults (n=7), respectively (Fig. 2A). The asymptotic values do not differ from each other (Kruskal–Wallis chi-squared=2.0, d.f.=2, P=0.36) but are slightly lower than the $\delta^{13}C$ isotopic ratio of the C4 diet ($\delta^{13}C_{diet}$=−17.0±0.7, juveniles: H_0=6.4, P<0.001, subadults: H_0=5.3, P=0.003, adults: H_0=4.3, P=0.04). The time required to exchange 50% of the carbon atoms in fat tissue with carbon atoms from the recently digested C4 diet (t_{50}) equalled 5.5±1.7, 2.1±1.3 and 3.6±2.1 h for the juveniles, subadults and adults, respectively. The t_{50} of subadult individuals is significantly shorter than that of juveniles (H_0=4.8, P=0.002; Fig. 2B, Table S2). The $\delta^{13}C_{breath}$ values followed a one-pool exponential incorporation model with $\delta^{13}C_{breath}(t)=-17.78-10.82e^{-0.31711t}$ for the juveniles, $\delta^{13}C_{breath}(t)=-18.12-9.09e^{-0.46395t}$ for the subadults and $\delta^{13}C_{breath}(t)=-17.68-11.24^{-0.23573t}$ for the adult individuals.

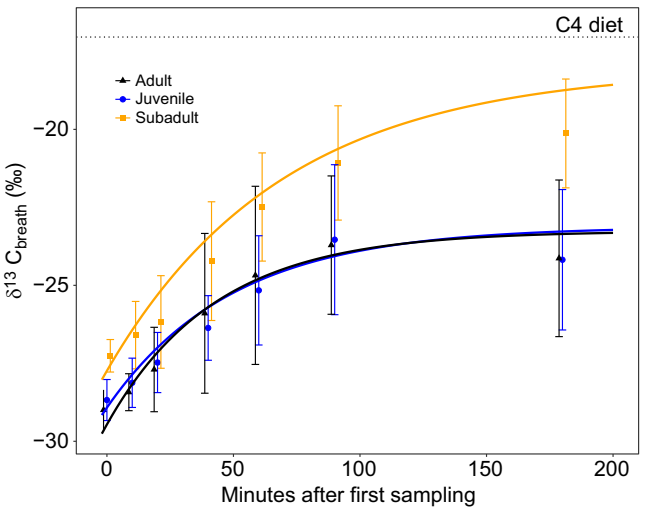

Fig. 1. Incorporation of C4 diet in the shrews' metabolism. Mean values of $\delta^{13}C_{breath}$ measured for 180 min from exhaled carbon dioxide after feeding on C4 diet in three different age/size classes. We calculated mean regression models based on averaged regression parameters. Error bars indicate s.d. Dotted line indicates mean $\delta^{13}C_{diet}$. At time 0, baseline sample $\delta^{13}C_{start}$ values differ significantly between subadults [N=7, body mass=7.06±0.67 g (means ±s.d.)] and juveniles (N=8, body mass=7.67±0.55 g) (H_0=5.3, P<0.001), and between subadults and adults (N=8, body mass=12.60±1.73 g) (H_0=5.2, P<0.001, see also Fig. S1A,C). Juveniles fuel 50% of their metabolism (t_{50}) within 44.9 min, subadults within 69.3 min and adults within 38.2 min (see also Fig. S1B).

Morphological parameters

Mean capture mass differed between age/size classes, with adults (12.60±1.73 g) significantly heavier than juveniles (7.67±0.55 g; H_0=4.6, P=0.003) and subadults (7.06±0.67 g; H_0=6.2, P<0.001) (Fig. S1C; Table S3). Mean standardised skull height differed between age/size classes, and was larger in juveniles (0.88±0.05)

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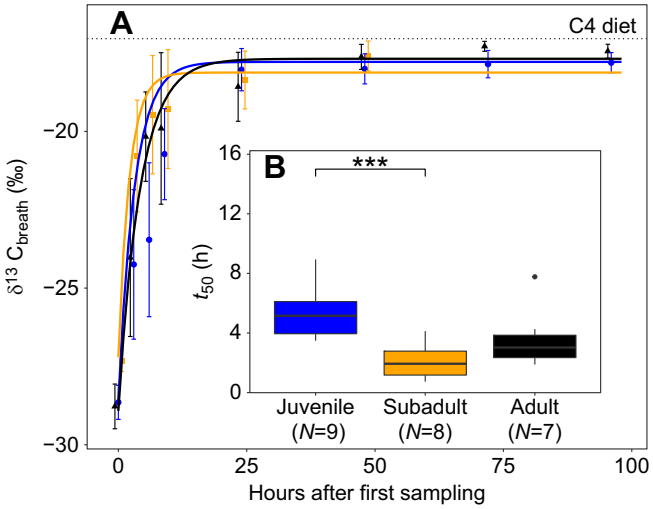

Fig. 2. Fat turnover experiment over 96 h. (A) Mean values of $\delta 13C_{breath}$ measured from exhaled carbon dioxide after feeding on C4 diet in three different age/size classes. We calculated mean regression models based on averaged regression parameters. The data from the 0 and 180 min samples are also presented in Fig. 1. Error bars indicate s.d. Dotted line: mean $\delta 13C_{diet}$. Juveniles replace 50% of their fat (t_{50}) after 5.5 h, subadults after 2.1 h and adults after 3.6 h. Asymptotic value is reached after 24 h. (B) t_{50} values for the three age/size classes for fat turnover experiment. There are significant differences between the juveniles and subadults in t_{50} (H_0=4.8, ***P=0.002). Box margins indicate the 25th and 75th percentiles, and solid lines within boxes the median. The outlier in the adults is indicated as a black dot. Sample sizes are given below each age/size class.

than in adults (0.79±0.04; H_0=3.9, P=0.002) and subadults (0.73±0.04; H_0=6.4, P<0.001), confirming that the shrews we tested showed Dehnel's phenomenon (Fig. S1D). We took each shrew's mass before and after each sample collection and calculated the mean of these measures and the changes in both body mass and the stable carbon isotope signature from one sampling to the next. We found a positive correlation between the body mass change (Δ body mass) and the change in $\delta^{13}C_{breath}$ of the shrews (ΔC) (ρ=0.415, P<0.001) (Fig. S2).

DISCUSSION

We measured the incorporation and fat turnover rate of wild-caught large summer juvenile, small winter subadult and regrown spring adult *S. araneus* by switching the shrews' diets to a differently labelled food source and monitoring the changes in the animals' $\delta^{13}C_{breath}$. We found that the incorporation rate remained rapid throughout the year and did not change between the age/size classes, even though the shrews undergo drastic anatomical and physiological changes (Dehnel's phenomenon). The shrews seemed to combust fat reserves and recently digested food simultaneously to fuel their metabolism. We found evidence that the shrews' natural diet changed between the seasons, reflected in differences in the stable carbon isotope baseline signature of their breath. Even though incorporation rate did not vary between age/size classes, we found that subadults, which are the age at which shrews are at their smallest, have significantly higher turnover rates than juveniles. In all three age/size classes, the whole-body fat was turned over between 9 and 24 h, showing that shrews are among those mammals with the lowest starvation tolerance. This supports the assumption that becoming smaller in the course of Dehnel's phenomenon may support the efforts of a highly metabolic mammal to save energy for survival under extreme environmental conditions.

Changes between the seasons in the shrews' natural diet

The differences between the $\delta^{13}C_{start}$ values are particularly interesting when referring to the shrews' natural diet, which is assumed to change in composition and quality between the seasons (Churchfield et al., 2012). Studies about shrews' natural diets show that these insectivorous animals require high-caloric and nutritious food (Gliwicz and Taylor, 2002). Yet, numerous prey species become unavailable in winter, e.g. earthworms, the main summer diet of shrews (Churchfield and Rychlik, 2006; Gliwicz and Taylor, 2002). Accordingly, it remains unclear how high the total energy uptake of shrews is during each season and how the diet changes in quality, even if the abundance of prey remains constant between seasons (Churchfield et al., 2012). For example, Churchfield et al. (2012) found that, in winter, the proportion of minute invertebrates (mainly diplopods) increases in the diet of *S. araneus*. Diplopods have a high content of indigestible chitin (Cummins and Wuycheck, 1971) and Churchfield suggested that the shrews only forage on these lower-quality food items when other, more suitable, prey is not available.

The differences we found in the baseline isotopic signature confirm the findings of Churchfield (2012). The natural diet of the shrews changes in composition and thus perhaps in quality with the seasons. In winter, the stable carbon isotope values are about 2‰ higher than during summer, indicating that either the shrews forage for a different kind of prey at least at our study site or that the shrews' prey consumes different plants with a higher C13:C12 ratio. However, better data on the seasonal composition of the shrews' diet are necessary to quantify how much energy is available to a shrew to fuel its metabolism and build up body fat reserves.

No differences in incorporation rate between the age/size classes

The sampling period for the incorporation rate experiment (Fig. 1) was 180 min and we found that, 60–90 min after the first C4 food ingestion, the $\delta^{13}C_{breath}$ converged close to an estimated asymptotic value. Within this time the shrews incorporated the recently digested food into their metabolism. In *S. araneus*, relative resting metabolic rate does not change between seasons (Taylor et al., 2013) and energy uptake is limited by the digestive tract physiology (Saarikko and Hanski, 1990). This explains why the incorporation rate did not differ significantly between our age/size classes (Fig. S1B), instead remaining consistently high throughout the year. This means that reported changes in digestive tract physiology (Jaroszewska and Wilczyńska, 2006; Kozlowska et al., 2004; Myrcha, 1967) are so well coordinated with the physiological processes that they affect food assimilation/incorporation rates to maintain a constant high level. Quality of our experimental diet did not change between seasons, which might not reflect changes in natural diet. However, the fact that we do not find differences between the seasons when feeding high-quality food further supports the hypothesis that the shrews' digestive tract already operates at the highest level and diet quality does not affect incorporation rates.

Nevertheless, comparing common shrews to other small animals such as *Noctilio albiventris* (lesser bulldog bat, body mass 23 g, feeding on mealworms) with a t_{50} of 27.3 min (Voigt et al., 2010), *Mus musculus* (house mouse, body mass 37 g, feeding on corn) with a t_{50} of 20.4 min (Perkins and Speakman, 2001), *Dendroica coronata* (yellow-rumped warbler, body mass 12 g, feeding on a mixture of simple carbohydrates, protein and fat) with a t_{50} of 264 min (Podlesak et al., 2005) or *Desmodus rotundus* (common vampire bat, body mass 30 g, feeding on blood) with a t_{50} of 29.5 min (Voigt et al., 2008b) shows that diet composition and body mass can influence incorporation rates (McCue and Welch, 2016;

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Voigt et al., 2008a). But, the effect of a different diet quality on incorporation rates in the same species undergoing such drastic changes as the ones caused by Dehnel's phenomenon has not yet been investigated and the relationship between all these factors remains unclear.

The shrews reached an estimated asymptotic value lower than the $\delta^{13}C$ value of the C4 diet we fed to them, which indicates that shrews continued to metabolise their limited fat stores or used other pathways to provide energy, such as liver gluconeogenesis, throughout the experiment.

Differences between individual incorporation rates were high and caused high standard deviations (Table S1). To get a more general view in each age/size class and to avoid misinterpretation driven by individual animals, we calculated mean values, which are less vulnerable to individual fluctuations and which should better reflect the overall status of shrews. For some individuals we were able to find a plausible explanation for diverging values. One example is individual 24 (adult), which had an exceptionally high incorporation rate. This individual consumed 1.63 g of food in 90 min, a relatively high amount, and yet it lost 0.47 g of mass during the same time. The metabolism of this individual may be very high for unknown reasons, letting it incorporate the food faster. Future studies should record overall or running wheel activity throughout the experiment. This might help to link a high metabolism to the potential behaviour of the animal.

Sorex araneus combust fat reserves and recently digested food simultaneously

Even though t_{50} did not differ significantly between age/size classes, the mean asymptotic $\delta^{13}C_{breath\infty}$ of the subadults was significantly higher compared with juveniles and adults. In general, the signature of the recently digested diet ($\delta^{13}C_{diet}$) differed significantly from the asymptotic $\delta^{13}C_{breath\infty}$, showing that *S. araneus* simultaneously use endogenous fat reserves while metabolising recently ingested food. This matches results from other animals using two sources to fuel their metabolism (Voigt et al., 2008a). In wild animals, fat stores are built from food digested before they were caught and the experimental food source usually does not match the stable carbon isotope signature of the natural food sources. Hence, $\delta^{13}C_{start}$, which reflects the stable carbon isotope signature of our shrews' natural diet, can cause the asymptotic $\delta^{13}C_{breath\infty}$ values to differ from each other while fat stores are combusted. We found similar differences between the age/size classes in $\delta^{13}C_{breath\infty}$ values and the $\delta^{13}C_{start}$ values (Fig. 2A), supporting this assumption. It still needs to be clarified under which circumstances the shrews tend to combust two energy sources, but one possible explanation is found in the positive correlation between changes in individual body mass and in $\delta^{13}C_{breath}$ values (Fig. S2). This shows that, when the animals lost body mass, the $\delta^{13}C_{breath}$ values decreased, which could mean that the shrews burned their fat deposits, especially when they did not eat for a while (spontaneous food intake varied greatly between individuals). At this point we are not able to determine which endogenous substrates were combusted, causing the depletion of the $\delta^{13}C_{breath}$ values, but measurements of $\delta^{13}C_{breath}$ values in combination with respirometry should reveal more insight about the shrews' metabolism and their energy source (Welch et al., 2006).

Differences in fat turnover rate between juvenile and subadult individuals

We measured the fat turnover rate of *S. araneus* over 48 or 96 h (Fig. 2A). Animals were food deprived 90 min prior to each breath sample collection after the first 90 min, to ensure they fuelled their metabolism only with endogenous substrate, i.e. body fat stores. All shrews reached the asymptotic $\delta^{13}C_{breath\infty}$, which differed significantly from the $\delta^{13}C_{diet}$ during this time. This is typical for animals that burn lipids to fuel their metabolism (Carleton et al., 2006). In general, the $\delta^{13}C$ signature of lipids is depleted compared to the carbohydrates from which they are synthesised (DeNiro and Epstein, 1977), and the $\delta^{13}C_{breath}$ therefore differed from the $\delta^{13}C_{diet}$ even when the animals reach the $\delta^{13}C_{breath\infty}$ and the body reserves were equilibrated to the new diet source. Comparing the t_{50} of the fat turnover rate between the three age/size classes revealed that subadult individuals had a shorter t_{50} compared with the juvenile individuals (Fig. 2B). The t_{50} from a fat turnover experiment can be used to infer how long the shrews would survive without food and was exceptionally low in subadult individuals (mean 2.1 h; Table S2), meaning that the shrews would have burned all fat reserves within 4.2 h. This matches the results of the experiments by Hanski (1994), who found survival times of food-deprived shrews to be 5–10 h. Nevertheless, these are only rough estimates for the starvation times, because also other body fuels such as proteins and carbohydrates will be metabolised before an animal dies (McCue, 2010). Another animal with high metabolism and low body mass, the broad-tailed hummingbird (*Selasphorus platycercus*), has a t_{50} of 0.8 days (19.2 h) (Carleton et al., 2006). Compared to this, especially the subadult shrews have exceptionally short t_{50} values. However, the shrews did not only have remarkably high fat turnover rates in winter, but also in the other age/size classes. Total fat was replaced between the sampling events after 9 and 24 h. *Sorex araneus* therefore depend on steady access to food year round, which explains that they have one of the lowest starvation tolerances recorded in mammals (McCue, 2012).

Our fat turnover experiment revealed large differences between individuals within each age/size class, causing high standard deviations (Table S2). Individual 20 (subadult), for example, had the highest fat turnover rate of less than 1 h. This individual had a relatively low capture body mass of 6.41 g, which could mean that this animal did not have large fat stores, which were then replaced very quickly. The same pattern is found in individual 13 (subadult), but a general statement about the correlation between body mass and fat turnover rate is not possible because the body mass of *S. araneus* can vary by 2 g within a day depending on recent food or water intake.

Possible link between changes in fat turnover and Dehnel's phenomenon in *S. araneus*

Dehnel's phenomenon, which causes size changes in most major tissues of the shrew to varying degrees, is thought to be an energy-saving strategy in winter (Churchfield, 2002; Gliwicz and Taylor, 2002; Hyvarinen, 1969; McNab, 1991; Pucek, 1970; Taylor et al., 2013). Using skull height, a commonly used proxy, we confirmed that our study animals followed the typical pattern of Dehnel's phenomenon. Standardised skull height decreased by 17.6% between summer juveniles and winter subadults and then showed a regrowth of 7.5% in spring adults (Fig. S1D, Table S3). Capture mass of our shrews also decreased by 9.9% from summer juveniles to winter subadults and then strongly increased by 45.6% when the animals became adults and reached sexual maturity (Fig. S1C, Table S3). The winter decrease in body mass is mainly caused by shrinkage of major organs (Churchfield, 1990; Mezhzherin, 1964; Pucek, 1970). The relative and absolute amount of BAT increases in winter (Pucek, 1965), and metabolic activity of the BAT also peaks in winter (Przełecka, 1981). This could help explain the higher fat turnover rate in the subadults, because these winter-caught shrews

might be burning their BAT faster to compensate for cold temperatures. Thus, although relative resting metabolic rates remain constant over the age/size classes (Taylor et al., 2013), fat turnover rate is more than twice as high in winter subadults compared with summer juveniles even though body mass does not decrease to the same extent. This seems to contradict the assumption that the Dehnel's phenomenon can help the animal to save energy, because the shrews would become more susceptible to food shortage in winter. By contrast, the high fat turnover rate and the resulting short survival time might be additional factors that increase the animal's need to reduce resource requirements and save energy in winter and thus may have at least contributed to the evolution of Dehnel's phenomenon, i.e. the reduction of overall size, and in particular expensive tissues, such as the brain and other organs. At this point, we are not able to tell which changes in the shrew's body are most important to save energy during winter, but future studies investigating changes in the shrew's whole body at different stages of Dehnel's phenomenon using non-invasive methods such as 3D imaging by computed tomography (Lubura et al., 2012) or the measurement of energetic properties of different tissues will help to shed more light on this question.

Our study expands our understanding of a unique and poorly known small-mammal wintering strategy. It also emphasises the influence of changes in the animal's environment, such as changes in the natural diet, confirming how stable isotopes can be used to address ecological questions. A simple and non-invasive experimental setup, generating samples of stable carbon isotopes of exhaled breath in a feeding experiment, can be used to address questions about an animal's physiology at different stages of the lifecycle, and we encourage researchers to consider this technique for more future studies testing hypotheses in organismal or comparative physiology.

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The authors declare no competing or financial interests.

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