

Minimally invasive collection of adipose tissue facilitates the study of eco-physiology in small-bodied mammals

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Summary

1. Adipose tissue is the primary fuel storage for vertebrates and is an important component of energy budgets during periods of peak energetic demands. Investigating the composition of adipose tissue can provide information about energetics, migration, reproduction and other life-history traits. Until now, most field methods for sampling adipose tissue of small-bodied vertebrates have been destructive. Therefore, investigations of adipose tissue in small-bodied vertebrates have been limited in their broadscale application.

2. We developed a field-ready micro-adipose biopsy method for sampling adipose tissue of small-bodied vertebrates, by adopting fine-needle adipose aspiration. We applied the method to silver-haired bats (*Lasionycteris noctivagans*) and then quantified the resulting fatty acid signatures of a summer group and an autumn group to demonstrate one possible application of the method.

3. We successfully obtained interpretable fatty acid signatures from 74.5% of micro-adipose biopsy attempts, with success positively correlated with body mass index. Summer and autumn groups of bats had different fatty acid signatures likely representing varied available dietary compositions at resident sites (the habitat where adipose deposits are accumulated prior to migration). The fatty acid profile of autumn silver-haired bats was largely characterized by 16:0, 18:1 and 14:0, and the summer group was characterized by 16:0, 16:1 and 18:0. Our results suggest that fatty acid signatures have the potential to characterize the origins of migrating individuals, or the number of unique subpopulations being supported by a migration route.

4. This field-ready fine-needle adipose aspiration method can be used on small-bodied mammals and modified for application to other small-bodied vertebrates. This non-destructive approach to sampling adipose tissue has great value because it allows for robust sample sizes, longitudinal studies of the same individuals across space and time, and sampling of rare, threatened and endangered species.

Key-words: bats, fatty acid signatures, fine-needle adipose aspiration, lipids, micro-adipose biopsy, movement ecology

Introduction

Adipose tissue is the primary fat storage tissue in vertebrates and acts as an energetic buffer during periods of peak energetic demand and food shortages (Karasov & del Rio 2007). It is also the primary fat storage tissue for birds and bats during seasonal migrations (Blem 1976; Alerstam & Lindstrom 1990; Hedenström 2009). Thus, adipose tissue plays an important eco-evolutionary role in the daily and annual energy budgets and physiology of many organisms.

Adipose tissue consists of triglycerides that are made up of three fatty acids esterified to a glycerol backbone. The unique composition of fatty acids present within an individual is informative of diet across space and time because fat is acquired directly from prey and remains mostly metabolically unaltered, though triglycerides may undergo modification and

biosynthesis (elongation and desaturation), *de novo* synthesis and differential mobilization (Morton & Lieberman 1974; Pierce & McWilliams 2005; Budge, Iverson & Koopman 2006; Price 2010). Fatty acid signatures can represent diet over the course of weeks to months, varying temporally and spatially due to the availability of exogenous fatty acid sources and individual life history and physiology (Budge, Iverson & Koopman 2006). For example, an organism that fasts during migration and exclusively metabolizes stored fuels should have a fatty acid signature that is representative of its resident site (its habitat prior to migration) where it acquired the adipose tissue. In this way, fatty acid signatures may be informative as geo-markers because individuals occupying the same resident sites have access to similar prey and presumably have similar fatty acid compositions. Identification of unique spatiotemporal groups using fatty acid signatures has been demonstrated in large seabirds, fishes and polar bears (*Ursus maritimus*), highlighting the sensitivity of fatty acid signatures to spatial and temporal variation in diet (Thiemann, Iverson & Stirling 2006; Iverson,

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Springer & Kitaysky 2007; Wang *et al.* 2009; Karnovsky, Hobson & Iverson 2012; Kim *et al.* 2014).

Studies involving adipose sampling of small-bodied vertebrates have used destructive methods (Bower & Helms 1968; Morton & Liebman 1974; Conway, Eddleman & Simpson 1994; Egeler & Williams 2000; Pierce & McWilliams 2005; McGuire, Fenton & Guglielmo 2013). For example, McGuire, Fenton & Guglielmo (2013) used destructive sampling to demonstrate differences in adipose tissue compositions between migrant and resident hoary bats (*Lasiurus cinereus*). Although destructive sampling can be informative, longitudinal sampling of individuals is obviously not possible and ethical concerns preclude robust sample sizes and sampling of species of conservation concern. Non-destructive techniques are widely used in studies of large-bodied taxa where adipose tissue is conspicuous and biopsies obtain relatively large quantities of adipose tissue (Thiemann, Iverson & Stirling 2006; Iverson, Springer & Kitaysky 2007; Strandberg *et al.* 2008). Presently, obtaining adipose biopsies is a challenge because traditional biopsy core sampling of small-bodied vertebrates is lethal and subcutaneous adipose tissue is far less conspicuous than in large-bodied vertebrates.

Fine-needle aspiration has been widely used in human and animal medicine as a safe and effective biopsy technique (Dixon *et al.* 1984; Hossein & Goellner 1993). Fine-needle adipose aspiration (FNAA) is a method used to obtain small volumes of adipose tissue for further sample analysis. For example, in human medicine it is used to sample the abdominal fat pad for amyloid detection (Blumenfeld & Hildebrandt 1993). As such, we reasoned that FNAA could satisfy the need for a minimally invasive, non-destructive technique to obtain micro-adipose biopsies from small-bodied vertebrates. Herein we demonstrate that FNAA is a safe and effective alternative to destructive sampling for a species of small-bodied insectivorous bat, the silver-haired bat (*Lasionycteris noctivagans*). By sampling both an autumn and summer population of silver-haired bats, we tested whether FNAA provided fatty acid signatures that were consistent with the results of McGuire, Fenton & Guglielmo (2013). Specifically, we predicted that an autumn group and a summer group of silver-haired bats would have differing fatty acid signatures.

Materials and methods

STUDY SPECIES

The migratory tree-roosting silver-haired bat (8–14 g body mass) was our focal species. Silver-haired bats are widely distributed across North America (Cryan 2003) and are opportunistic predators that consume a variety of insects (Kunz 1982).

STUDY SITES

We captured bats at a summer site, composed of resident individuals and an autumn site composed of migratory individuals that presumably use the site as a stopover or wintering ground (Weller & Stricker 2012). Due to their geographic proximity (400 km), we cannot rule out intermixing of some individuals between the summer and autumn sites.

At the summer site, silver-haired bats were captured on nine occasions from 17 July to 13 August, 2014, using mist nets set across Willard Creek, Lassen National Forest, Lassen County, California, USA (latitude: 40.367240, longitude: –120.802824). At the autumn site, we attempted captures on 35 nights from 27 August to 29 October 2014, using mist nets set across Bull Creek, Humboldt Redwoods State Park, Humboldt County, California, USA (latitude: 40.352316, longitude: –123.985523). At the autumn site, abundance of silver-haired bats is lowest during summer and limited primarily to males (Weller & Stricker 2012).

FINE-NEEDLE ADIPOSE ASPIRATION

Upon capture, we measured forearm length (± 0.1 mm), body mass (± 0.1 g), sex, reproductive status and age. We sampled adipose tissue of adult silver-haired bats that were >9 g. During the summer resident period, we did not sample females that were pregnant or lactating.

We identified common sites of adipose tissue deposits by internally inspecting carcasses obtained from wildlife rehabilitation centres and submitted to museums for specimens. The most consistently abundant adipose deposit on the carcasses was located on the caudolateral dorsum region of the bats (Fig. 1a). Fortunately, the lateral and dorsal position of this adipose deposit facilitated restraint and sampling of bats in the field.

We extracted micro-adipose samples (<0.2 mg) from the caudolateral dorsum using FNAA on live bats in the field. To ensure safety of the bat, two people completed the procedure in the field. One person secured the bat while the other person obtained the fat sample. The bat was held securely, ventral side down, against a small piece of plastic processing surface, using a cloth bag pinched taut on both sides of the top half of the body for restraint (Fig. 1a).

The person performing the FNAA gently extended each of the bats legs to a 45° angle away from the body and placed a piece of medical tape across the knee joint (Fig. 1a). We palpated bats to determine quantity of subcutaneous adipose present in the lower dorsum region. We developed a palpation guideline to identify the adipose deposit and the needle insertion site. We used the spine, pelvis and abdominal wall (Fig. 1b) as palpation guidelines in the field to help locate the adipose deposit and needle insertion site. We identified the needle insertion site as the point just above the hip of the bat at the caudal limit of the adipose tissue deposit (Fig. 1b). If we could not identify the adipose tissue via palpation, due to poor body condition of the bat, we did not attempt to collect an adipose sample from that individual.

Once we identified the adipose deposit and needle insertion site, we cleaned the caudolateral dorsum of the bat with isopropyl alcohol and used our free hand to lift and pinch the skin away from the body creating a 'tent' at the needle insertion site (Fig. 1b). We then inserted the needle (22G $\times$ 1) with the bevel up and then rotated it 180° such that the bevel was facing down and directed parallel to the abdominal wall. We then progressed the needle downward into the adipose tissue. We pulled back on the plunger of the syringe (5 cc) creating negative pressure and moved the needle back and forth in the adipose tissue as many as six times or until we could see adipose tissue in the hub of the needle. The entire process lasted *c.* 90 s. Bats were released at the capture site immediately following the procedure. To avoid oxidation, we expelled samples into a PTFE-lined screw-cap 1-mL micro-reaction vial (Wheaton, Millville, NJ, USA) which were then placed on dry ice until transported to a -80°C freezer.

All protocols were approved by the Humboldt State University Institutional Animal Care and Use Committee (Protocol

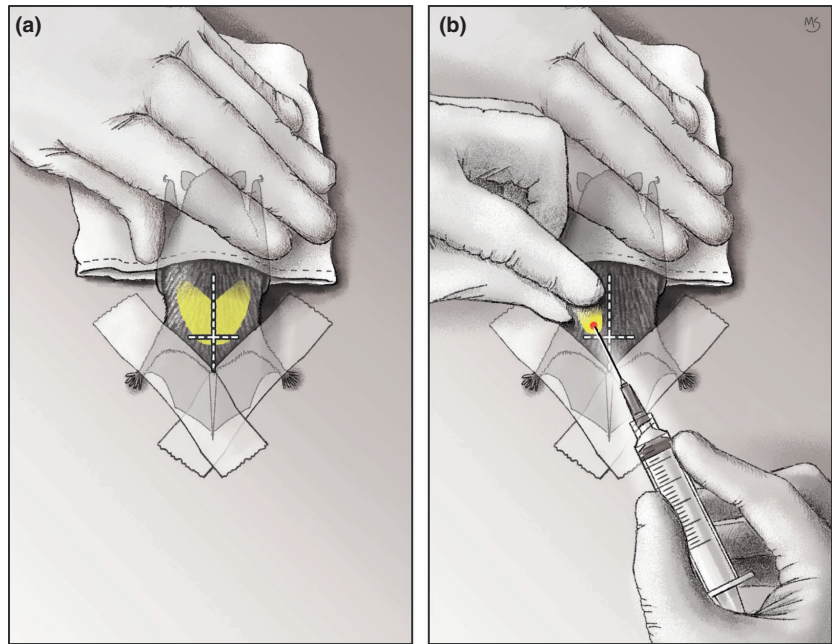


Fig. 1. (a) Dorsal view of a bat being restrained by medical tape across its legs and held in a cloth bag prior to fine-needle adipose aspiration showing the adipose deposit (yellow shading) in relation to the spine (vertical dashed line) and pelvis (horizontal dashed line). (b) Dorsal view of bat showing the lower lateral adipose deposit (yellow shading) and the needle insertion site (red dot). The person performing the fine-needle adipose aspiration (22-gauge needle) 'tents' the skin with one hand while using the syringe with the other. Illustration by: Meg Sodano.

no. 13/14.B.103-A). The study was conducted under California State Fish and Wildlife scientific collections permit no. 013013.

Fine-needle adipose aspiration compares well in procedure and required skill level to injecting a passive integrative transponder (PIT) tag subcutaneously into a small mammal. The method of inserting a needle into the subcutaneous space is similar with both techniques with the biggest difference being that PIT tag needles are generally much larger (12 gauge). Research conducted on the effects of using needles on small mammals have found no long-term injuries or decreases in survival rate associated with the insertion of PIT tags (Neubauer *et al.* 2005; Ellison *et al.* 2006).

ADIPOSE TRANSESTERIFICATION, PHASE SEPARATION AND GC-MS ANALYSIS

For adipose transesterification and phase separation, we used a sub-microscale *in situ* method (Bigalow *et al.* 2011). Briefly, frozen samples were placed in a Centrivap (Labconco, Kansas City, MO, USA) at 35 °C for 3 h to remove water present in the sample. Afterwards, 200 µL of analytical-grade methanolic boron trifluoride (1.3 M) (Sigma-Aldrich, St. Louis, MO, USA) was added to each sample, and samples were flushed with nitrogen and then vortexed for 10 s. We then placed the vials in a heat block for 1 h at 100 °C. After the vials cooled, we added 250 µL of analytical-grade isooctane (Sigma-Aldrich), followed by 500 µL of reagent grade sodium chloride (Sigma-Aldrich). In between the addition of isooctane and sodium chloride, we flushed each sample with nitrogen for 10 s and vortexed each sample for 10 s. The sample layers separated after the final vortexing, with the isooctane containing the fatty acid methyl esters (FAMES) in the top layer. We then transferred the FAME containing isooctane to gas chromatography–mass spectrometry (GC-MS) vials (Varian, Palo Alto, CA, USA). We added sodium sulphate (Agilent, Santa Clara, CA, USA) to dry the sample. We then began GC-MS analysis using 1 µL split-less injections of our sample.

We performed the GC-MS analysis on a HP 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) and a HP 590 mass spectrometer (Hewlett Packard). We used a

30 m × 0.25 mm × 0.20 µL HP-88 high-resolution polar gas chromatography column (Agilent). Both the injector and detector temperatures were set at 250 °C. The method started by holding an initial temperature of 50 °C for 1 min. We then increased the temperature by 45 °C min⁻¹ to 153 °C where it held the temperature for 2 min. We then increased temperature by 5 °C min⁻¹ until it reached the final temperature of 230 °C where it held for 2 min. We identified FAMES by comparing the retention time of the samples with the retention times of a 28-component external standard (NLEA FAME mix; Restek, Bellefonte, PA, USA).

We identified 15 fatty acids in our analysis and eliminated fatty acids that were <14 carbons long and accounted for <1% of the total fatty acid composition. We omitted one unidentified fatty acid constituent that we were unable to match with our external standard that fell in the range of 18:3, 22:0, 22:1, 24:0, 20:5 and 24:1. We considered the following fatty acids in our analyses: 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 20:0, 20:1.

STATISTICAL ANALYSIS

We normalized our abundance values from the GC-MS results and calculated the total proportion all nine fatty acids considered. Since fatty acid signature data were not normal, we transformed the fatty acid proportions for the analysis as recommended by Budge, Iverson & Koopman (2006) by applying an additive log ratio transformation from Aitchison (1986): $X_{\text{transform}} = \ln(x_i/Cr)$, where $X_{\text{transform}}$ is the transformed fatty acid data, x_i is a given fatty acid in the data set expressed as a proportion of total fatty acid composition, and Cr is the percentage of a within-data set reference fatty acid. We used 18:0 as our reference fatty acid as recommended by Budge, Iverson & Koopman (2006).

We calculated body mass index (BMI) for each individual as mass/forearm length and compared mean BMI between groups using a *t*-test. We then correlated BMI with FNAA success using logistic regression. We used a pairwise multivariate analysis of variance (MANOVA) to assess differences among proportions of individual fatty acids between individuals captured during summer and autumn. We used a non-metric multidimensional scaling (NMDS) approach to ordinate and

graphically represent and assess the Bray–Curtis distances between individuals captured during summer and autumn. Bray–Curtis distances are bound between 0 and 1 and indicate the dissimilarity of fatty acid signatures between two individuals such that a value of 0 would indicate an identical signature. The analysis was completed with the function metaMDS in the R package Vegan (Oksanen *et al.* 2013). We tested for differences between group (summer and autumn) and sex using a nonparametric, permutational multivariate analysis of variance (PERMANOVA) of the Bray–Curtis distances. We also tested intra-group differences by sex. The analysis was completed with the function adonis in the R package Vegan (Oksanen *et al.* 2013). All statistical analyses were conducted in the R programming environment (version 0.98.501; R Core Team 2013), and all values are reported as mean $\pm$ SE.

Results

We successfully applied FNAA in the field. The application of the method was quick and minimally invasive. Bats showed no signs of injury or distress and behaved normally upon release. We performed a total of 114 FNAs. The adipose aspirations resulted in 85 (74.5%) quality micro-adipose biopsies from which we could derive interpretable fatty acid signatures. Of the 29 micro-adipose biopsies that did not produce interpretable results, 15 (13.1%) were dilute and lacked sufficient adipose for detection during analysis, 10 (8.7%) samples were eliminated because they had visible blood contamination, and four (3.5%) samples were both dilute and blood contaminated. Samples that were composed of more blood than adipose produced total ion chromatograph results that had drastically different fatty acid compositions compared to the samples that had no blood present (visual inspection, Fig. S1b, Supporting Information). We removed all samples that were blood contaminated. Dilute samples produced total ion chromatographs that were indiscernible from background (Fig. S1c) and were also removed. Mean BMI of the summer group was

significantly lower than mean BMI of the autumn group (summer: 0.25 ± 0.003 , autumn: 0.29 ± 0.003 ; $t_{98.91} = -7.33$, $P < 0.0001$), and micro-biopsy success was positively related to BMI (SE: 13.43, z -value: 3.88, $P < 0.001$).

In silver-haired bats, the fatty acids 18:1, 16:0 and 16:1 accounted for an average of 0.72 ± 0.34 of the proportion of fatty acids among the autumn individuals and 18:1, 16:0 and 14:0 accounted for an average of 0.79 ± 0.06 of the proportion of fatty acids among summer individuals. We found differences in the proportion of each of the individual fatty acids between the autumn and summer group except for 18:1 and 20:0 (Fig. 2). The greatest differences were in 16:0 (autumn: 0.24 ± 0.01 , summer: 0.34 ± 0.02 ; $F_{1,83} = 19.92$, $P < 0.0001$) and 14:0 (autumn: 0.07 ± 0.01 , summer: 0.16 ± 0.02 ; $F_{1,83} = 20.31$, $P < 0.0001$). Using NMDS ordination, we observed a distinct group of fatty acid signatures in individuals captured at the autumn site, but values for some individuals overlapped with individuals from the summer site (Fig. 3). The NMDS stress value was 0.147, indicating that our two-dimensional representation of the data was at an acceptable and interpretable level. We confirmed group differences between the autumn and summer groups (ADONIS, $F_{1,83} = 17.86$, $P < 0.0001$) and found no effects of sex (ADONIS, $F_{1,83} = 1.00$, $P = 0.377$). We did not detect within-group differences between sexes in fatty acids in either the autumn group (ADONIS, $F_{1,57} = 0.40$, $P = 0.7997$) or the summer group (ADONIS, $F_{1,24} = 1.73$, $P = 0.149$).

Discussion

Fatty acid signatures have wide application and potential for addressing eco-physiological questions in mammals. However, until now, analysis of fatty acid signatures has been limited to large animals or destructive sampling. We demonstrated that

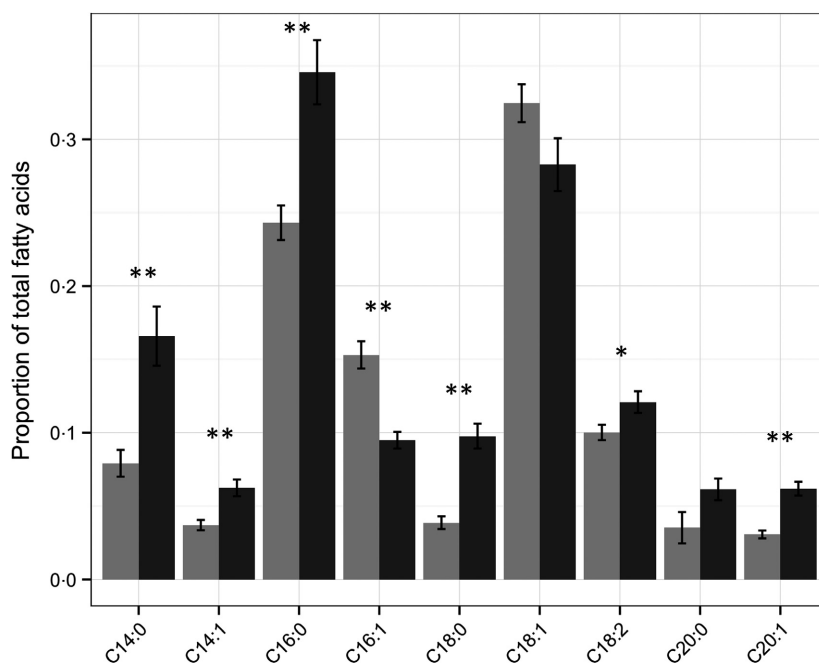


Fig. 2. Proportion of individual fatty acids calculated from fine-needle adipose aspiration samples taken from silver-haired bats captured during autumn in Humboldt Redwoods State Park, Humboldt County, California, USA (light bars, $n = 59$), and during summer in Lassen National Forest, Lassen County, California, USA (dark bars, $n = 26$). Bars indicate means $\pm$ SE and significance levels calculated by pairwise MANOVA. Data were log-transformed following Aitchison (1986). * $P < 0.05$, ** $P < 0.001$.

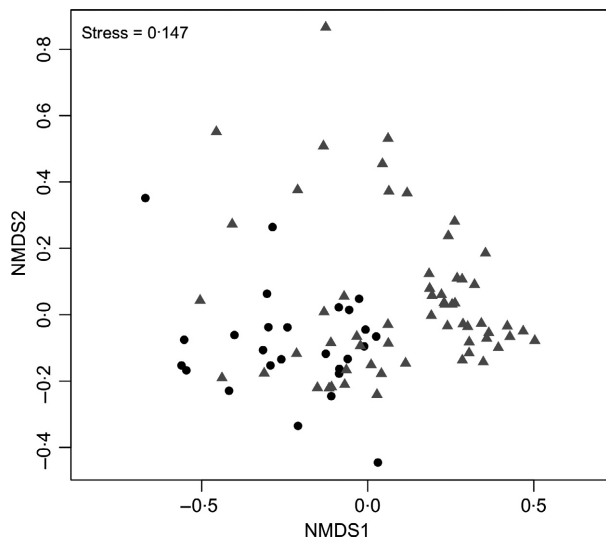


Fig. 3. Bray–Curtis non-metric multidimensional scaling (NMDS) ordinations of fatty acid signature composition data for micro-adipose biopsy samples taken from silver-haired bats captured in Humboldt Redwoods State Park, Humboldt County, California, USA, during autumn (grey triangles, $n = 59$) and Lassen National Forest, Lassen County, California, USA, during summer (black circles, $n = 26$). Data were log-transformed following Aitchison (1986).

FNAA is a safe method of extracting micro-adipose samples from live bats in the field. We also demonstrated an application of the method by quantifying differences in fatty acid signatures between silver-haired bats captured in the summer and autumn.

Overall, we were encouraged with our FNAA success rates and would expect improvement with further practice in the field. We did not observe adverse reactions from bats indicative of undue levels of stress (e.g. excessive bleeding or reluctance to take flight upon release). Similarly, in other work we have collected FNAA from big brown bats (*Eptesicus fuscus*) and have had equal success in obtaining and analysing micro-adipose samples (data not presented here). Two classes of samples obtained from silver-haired bats resulted in non-interpretable data: samples that were dilute and those that were blood contaminated. Dilute samples were associated with lower BMI; thus, seasonal variation in body condition must be considered when projecting potential FNAA success rates because silver-haired bats had a greatly reduced BMI during summer.

One potential limitation of FNAA is the potential for heterogeneous adipose deposits. Because only a small portion of adipose tissue is used for analysis, it is important to ensure samples obtained via FNAA are representative of the fat deposit as a whole. Generally, fat deposits of terrestrial mammals are assumed to be homogenous (Budge, Iverson & Koopman 2006) and homogeneity in fat has been confirmed in mink (*Mustela vison*) (Layton, Rouvinen-Watt & Iverson 2000) and polar bears (Thiemann, Iverson & Stirling 2006).

We found differences in fatty acid composition between bats sampled during summer and autumn. Since diet likely drives the differences in adipose composition, it follows that

the observed differences between summer and autumn sampled individuals could be due to temporal shifts in diet, geographic isolation (i.e. differential prey availability across unique resident habitats) or a combination of both. We expect to see temporal shifts in diet leading to changes in fatty acid signatures over the course of summer residency due to naturally changing availability of insect prey, but it is unknown how rapidly fatty acid composition of an individual changes over time. Furthermore, it is unlikely that the rate of change in fatty acid composition is constant (Wang *et al.* 2009). In fact, we would expect rapid changes occur during the resident period followed by more static rates during migration. The reduction of digestive organs during the migratory period observed in hoary bats, along with the use of torpor-assisted migration observed in silver-haired bats (McGuire, Jonasson & Guglielmo 2014), suggests that tree bats minimize the time spent foraging during migration compared to the resident period, and likely reduce rather than increase their fat stores during migration (McGuire, Fenton & Guglielmo 2013). The fact that individuals sampled during the summer had a lower BMI than individuals sampled during the autumn indicates that silver-haired bats are likely hyperphagic leading up to the autumn migration which is consistent with short-distance migratory hibernating bats (Fleming & Eby 2003). The geographic proximity between our study sites coupled with some individuals from the autumn site exhibiting fatty acid compositions similar to those measured at the summer site suggests there could be some intermixing between these populations. Future research comparing fatty acid signatures of individuals inhabiting multiple unique geographic locations throughout the summer residency period will provide a critical next step and perhaps disentangle spatial and temporal variability in fatty acid signatures.

Using destructive sampling, McGuire, Fenton & Guglielmo (2013) found that fatty acid signatures of hoary bats varied between spring migration and summer residency periods. Using this new non-destructive method, we generated comparable findings for silver-haired bats in that residents and migrants had unique fatty acid signatures. However, unlike McGuire, Fenton & Guglielmo (2013), we did not detect sex-specific differences in fatty acid composition. This is likely due to differences in life-history traits between spring and autumn migration. During autumn migration, both male and female tree bats use torpor-assisted migration as an energy saving strategy, greatly reducing the need to forage (Cryan & Wolf 2003; McGuire, Jonasson & Guglielmo 2014). McGuire, Fenton & Guglielmo (2013) collected samples in the spring when only male tree bats are expected to use torpor-assisted migration while female tree bats use a homeothermic migration strategy due to the thermoregulatory constraints associated with migrating during active pregnancy (Cryan & Wolf 2003). Therefore, fatty acid signatures should differ between males and females in the spring.

We applied FNAA in the context of migration ecology to demonstrate one possible application of the technique. Our results suggest that fatty acid signatures have the potential to

characterize the origins of migrating individuals, or the number of unique subpopulations being supported by a migration route (uniqueness). Certainly this method could be applied in different contexts as well, such as reproductive physiology, diet and adipose soluble contaminants. In addition, analysis of fatty acid signatures could provide complimentary data sets to be coupled with other non-destructive analyses [e.g. stable isotope analyses (Wunder 2010)] to infer migration routes.

A field-ready micro-adipose biopsy method, FNAA, can be applied broadly to bats and easily adapted to other small-bodied vertebrates. Since FNAA is non-destructive and can be applied to small-bodied vertebrates, new research avenues that require robust sample sizes repeated sampling of the same individual through time, and sampling endangered and threatened species is now possible. Using FNAA to characterize fatty acid signatures can tell us about the adipose tissue composition and the physiological interplay with seasonal food availability, which can then be used in studies of energetics, migration, reproduction and other life-history traits.

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Data accessibility

Data deposited in the Dryad repository: <http://dx.doi.org/10.5061/dryad.b859k> (Clerc et al. 2016).

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. The total ion current spectrograph of a successful, blood-contaminated, and dilute adipose sample.