Comparative Biochemistry and Physiology, Part B 274 (2024) 111008

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Commentary: Tracing the fate of metabolic substrates during changes in whole-body energy expenditure in mice

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ARTICLE INFO

Edited by Chris Moyes

Keywords:
Open-flow respirometry
Radiolabelled isotopes
Positron emission isotopes
Stable isotopes
^{14}C-2-bromopalmitic acid

ABSTRACT

For small mammals, such as mice, cannulation procedures can be quite challenging, limiting research associated with tracing isotopically labelled substrates at the whole-animal level. When cannulation in mice is possible, assessment of substrate use is further limited to when mice are either under anesthesia or are at rest, as there are no studies directly quantifying substrate use during exercise in mice. The use of isotopic tracer techniques has greatly advanced our knowledge in understanding how metabolic substrates (carbohydrates, amino acids, and fatty acids) contribute to whole-body metabolism. However, research regarding tissue-specific fuel use contributions to whole-body energy expenditure in mice at varying metabolic intensities (i.e., exercise) is lacking, despite the popularity of using mice in a variety of metabolic models. In this commentary, we briefly discuss the methodologies, advantages, and disadvantages of using radiolabelled, positron emission, and stable isotopes with a specific focus on fatty acids. We highlight recent mouse studies that have used creative experimental designs employing the use of isotopic tracer techniques and we briefly discuss how these methodologies can be further pursued to deepen our understanding of substrate use during exercise. Lastly, we show findings of a recent study we performed using a radiolabelled fatty acid tracer (^{14}C-bromopalmitic acid) to determine fatty acid uptake in 16 muscles, two brown and two white adipose tissue depots during submaximal exercise in deer mice.

1. Introduction

Indirect calorimetry is widely a used technique to assess whole-body energy metabolism, to predict proportional use of metabolic fuels, and to estimate absolute rates of substrate oxidation (Frayn, 1983; Ferrannini, 1988; Lighton and Halsey, 2011). Whole-body energy metabolism can be sustained with various patterns of fuel use (oxidation of carbohydrates, fatty acids, and amino acids) and decades of studies on exercise metabolism using indirect calorimetry have determined how fuel use varies with work intensity, in humans (Brooks and Mercier, 1994) and across other mammal species (Schippers et al., 2014). This technique has demonstrated that in mammals, as exercise intensity increases, the relative proportioning and absolute reliance on glucose increases, while the relative proportioning of lipids decreases. The validity of indirect calorimetry measurements relies on several assumptions, such as the organism being in steady-state and that the contribution of protein oxidation to total energy is considered 'negligible' during conditions such as exercise (Mtaweh et al., 2018). Furthermore, indirect calorimetry cannot distinguish which tissues contribute to whole-animal rates of substrate oxidation. Quantifying the contributions of these fuel sources to whole-body energy metabolism requires using isotopic tracer techniques that can directly assess in vivo tissue uptake and oxidation of substrates. It is worth nothing these types of tracer experiments differ from the use of isotopically labelled metabolites in metabolic flux experiments, which trace the movement of label through biochemical pathways of specific tissues (e.g., Jang et al., 2018; Hui et al., 2020; Midha et al., 2023; McBride et al., 2024).

Isotopic tracers have greatly advanced our understanding of the contributions of specific metabolic substrates to whole-body energy metabolism and is a topic that has been extensively reviewed (Murphy, 2006; McCue, 2011; Welch Jr et al., 2016). However, barriers such as tracer costs and/or access to specialized equipment may dissuade researchers from pursuing isotopic tracer studies. For example, a small animal Positron emission tomography (PET) scanner can cost between

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https://doi.org/10.1016/j.cbpb.2024.111008
Received 30 April 2024; Received in revised form 20 July 2024; Accepted 22 July 2024
Available online 24 July 2024

$400,000 and $1,200,000 (Yao et al., 2012). In addition, despite the popular use of mice as experimental models (Laughlin et al., 2012), quantification of substrate fuel utilization using isotopic tracers is lacking, particularly for our understanding of fuel use contributions to conditions other than rest, (i.e., during exercise, thermogenesis, or torpor, etc.; McCue and Welch, 2016). This lack of research may be due to the challenges associated with working with a small animal, such as surgical catheterization necessary for certain tracer techniques. The popularity of the continuous infusion technique was established using relatively large animals, from rats (i.e., McClelland et al., 1999) to humans (i.e., Jensen et al., 1989; Brooks, 1998). Techniques such as continuous infusion of isotopically labelled substrates, combined with serial blood sampling and tissue biopsy, has allowed for the quantification of appearance into and disappearance of fuels from the circulation, and potentially substrate oxidation, in a variety of species in various metabolic states. However, these experiments were possible, in part, due to the ease of cannulation and tissue biopsies in larger animals. Cannulating mice requires highly precise microsurgery by trained personnel, a technique only a few labs employ, or which can be made for purchased by various vendors (i.e., Jackson Laboratories and Charles River Laboratories). Furthermore, while it is possible to infuse and collect serial blood samples at rest in mice, (Hughey et al., 2014; Hui et al., 2017; Park et al., 2018; Faubert et al., 2021; Zhang et al., 2021), it is very challenging to do so if the small animal is either exercising, or in hibernation/torpor, without disturbing the animal's natural behaviour. Moreover, the ability to collect tissue biopsies in vivo is limited in mice, and tissue collection at specific endpoints requires sacrificing individual animals. So, while mice are currently the preferred experimental model used in a variety of studies and across many disciplines to understand changes in energy demand and fuel use, there is a lack of research which directly assesses tissue substrate use with increasing (i.e., exercise) or decreasing (i.e., torpor) metabolic demands.

There are obvious limitations of using isotopic tracers to assess fuel use in mice; however, the advantages of these techniques have led to the development of creative experimental designs employing isotopic tracers (i.e., Martins et al., 2002; McCabe and Previs, 2004; McCue and Pollock, 2013). In this commentary, we briefly review the uses, advantages and limitations of using isotopic tracers, with an emphasis on those who study fatty acids as a way of assessing lipid metabolism in mice. We highlight recent research using radiolabelled, positron emitting, and stable isotopes for assessing lipid metabolism in mice, and comment on how these techniques and methodologies can be applied to use during exercise. Lastly, we provide an example of a case study that traces the fate of fatty acids during exercise in lab born and raised deer mice.

1.1. Isotopic tracers

The appearance of metabolic substrates into to the circulation upon release from storage depots, and subsequent uptake and oxidation in active tissues, has relied on the use of isotopic tracers (Kim et al., 2022; Carpentier, 2024). Metabolic tracer studies employ substrates with radiolabelled isotopes (e.g., ^{14}C and ^{3}H), positron emitting isotopes (e.g., ^{18}F and ^{11}C), and stable isotopes (e.g., ^{13}C and ^{2}H). These tracers enable the assessment and/or visualization of substrate turnover and/or oxidation at the whole-animal, circulation, and tissue level. Furthermore, differences in the kinetics of orally administered and intravenously administered tracers provides crucial information regarding nutrient oxidation during their first pass through the gut as demonstrated in variety of species, such has birds, lizards, mice, and humans (McCue et al., 2011, 2013; Plasman et al., 2019; Zeng et al., 2022; de Souza et al., 2023). Historically, protocols for isotopic tracer studies have been used more routinely to evaluate glucose and amino acid metabolism, relative to studies on lipids, likely due to the complexity of lipid metabolic pathways and diverse role in physiological function.

1.2. Radiolabelled isotopes

The use of radiolabelled isotopes offers a highly detectable and sensitive tracer assessment within the blood and in tissues (de Lima, 1998). These isotopes have been used for several decades to quantify tissue substrate uptake and oxidation, mainly in larger mammals (Evans et al., 1963; Yamdagni and Schultz, 1969; McClelland et al., 1999) and humans (Jensen et al., 1989; Brooks, 1998). When paired with indirect calorimetry, biochemical, and molecular tissue analysis, one can attain important information regarding not only the oxidation of specific substrates, but also the contributions of circulation and intracellular sources to total oxidation rates. For example, a series of elegant experiments determined carbohydrate and fatty acid use in highly aerobic dogs compared with more sedentary goats, at rest and during exercise. In these experiments, indirect calorimetry was used to quantify whole-animal lipid oxidation rates (Roberts et al., 1996) and the continuous infusion of 1-[^{14}C]-palmitic acid and ^{14}C-sodium bicarbonate via the carotid artery, and venous blood sampling via the jugular vein was used to quantify circulatory fatty acid oxidation (Weber et al., 1996). By subtracting the rate of circulatory lipid use from the whole-animal lipid oxidation rate, they were able to estimate the contribution of intramuscular lipid oxidation during exercise, which was verified using histological analysis to assess lipid droplet size in the muscle (Vock et al., 1996; Weber et al., 1996). These studies were novel because they were able to quantify the contribution of circulating fatty acids and intracellular triglycerides to varying levels of locomotory demand and how they vary with differences in aerobic capacity. Despite dogs and goats being considerably larger than mice, some work has demonstrated the use of radioisotopes and cannulation to assess substrate oxidation in mice at rest. A recent study (Schönke et al., 2018) developed a protocol to assess whole-body fatty acid oxidation in conscious mice by infusing ^{3}H-palmitic acid and ^{14}C-2-bromopalmitic acid (non-oxidizable fatty acid), taking serial blood samples over time and collecting tissues at a set time following isotope infusion. They were able to quantify whole-body fatty acid oxidation and tissue-specific fatty acid uptake in multiple individual tissues. While this technique could not directly assess tissue-specific oxidation, nor could it easily be used if a mouse was undergoing exercise, where serial blood sampling would be very challenging, it provides the best example of how fatty acid metabolism can be assessed in mice under a variety of metabolic conditions (i.e., fasting vs fed state, therapeutic interventions, thermal acclimations, etc). While the use of radioisotopes has greatly advanced our understanding of energy metabolism and fuel use, it has its disadvantages. Given their radioactivity, working with radioisotopes requires many safety precautions to minimize researcher exposure, high quality training is necessary, and institutional and regulatory permits are required to ensure and maintain a safe work environment, which may be problematic depending on research facilities. Radiolabelled tracers can also be expensive and necessitate access to equipment to quantify radioactivity by scintillation counting.

1.3. Positron emitting isotopes

PET-computed tomography (PET-CT) produces images based in detection of positron emitting radiotracers to visualize and quantify changes in blood flow, tissue substrate uptake, and tissue metabolism (Mammatas et al., 2015). While PET-CT has been used to diagnose metabolic disorders (cancer, heart disease, etc), it has also greatly improved our understanding of tissue substrate use. ^{18}Flurodeoxyglucose (^{18}FDG) is a commonly used positron-emitting radiotracer employed to identify tissues with significant glucose uptake, while ^{18}Fluro-6-thia-heptadecanoic acid (^{18}FTHA), a fatty acid that is transported and taken up by tissues like other common fatty acids (i.e., palmitic acid), has been used to visualize tissue fatty acid uptake (further detailed in Takala et al., 2002). On its own, ^{18}FTHA (administered either intravenously or orally) can be used to assess tissue distribution of fatty

acid uptake (Labbé et al., 2011). When ^{18}FTHA is taken up by the tissue and is destined for fatty acid oxidation, it enters the mitochondria to undergo β-oxidation; however, the sulfur substitution in ^{18}FTHA prevents further breakdown, trapping it within the mitochondria and providing a metric of fatty acid oxidation. When ^{18}FTHA is paired with ^{11}C-acetate (a molecule used to assess tissue oxidative metabolism), it can provide a powerful visualization tool for assessing tissue-specific fatty acid uptake and oxidation. Most work using ^{18}FTHA has been done to visualize and assess fatty acid uptake and β-oxidation across a variety of tissues in humans (Mäki et al., 1998; Din et al., 2018; Saari et al., 2020), pigs (Takala et al., 2002), and rodents (DeGrado et al., 1991; Ci et al., 2006; Zhang et al., 2018; Nyrén et al., 2023). In terms of its general use, ^{18}FTHA has been mainly used in humans and less so in small mammals. In terms as a diagnostic visualization tool, ^{18}FDG has been used more widely across humans and other mammals. In a study of lean and obese mice, ^{18}FDG was administered via the tail vein followed by 1 h of moderate exercise. Glucose uptake into the muscle was then quantified using PET-CT (Miyatake et al., 2020). This same technique could also be applied using ^{18}FTHA to visualize muscle fatty acid uptake during exercise. While ^{18}FTHA has been used in the assessment of fatty acid uptake into oxidative and metabolically active tissues (heart, liver, muscle, kidney, brown adipose tissue), one study reported that ^{18}FTHA uptake and retention was low and may be inconsistent in the visualization for certain fat depots in mice (Zhang et al., 2018). A further limitation of these techniques is the use of anesthesia to facilitate administration of the tracer and to keep mice immobilized during PET-CT scanning. The use of anesthesia has the potential to disrupt physiological mechanisms associated with substrate use and should be taken into consideration when assessing the generalizability and physiological relevance of findings (Constantinides and Murphy, 2016). Overall, ^{18}FTHA can serve as a powerful fatty acid uptake visualization tool to address questions relating to the metabolic status of the organism.

1.4. Stable isotopes

The use of stable isotopic fatty acid tracers is extremely useful in the assessment of fuel utilization within biological systems. Research using stable isotopes has become increasingly popular when compared to radioisotopes, given the broader availability of mass spectrometry techniques and infrastructure (i.e., isotope-ratio mass spectrometry, cavity ring-down spectroscopy) and greater safety to animals and researchers compared with radioisotopes. In brief, the principle behind stable isotopic tracers involves the introduction of an isotopically labelled tracer (i.e., ^{13}C-palmitic acid, ^{2}H-palmitic acid, etc) either through dietary intake of labelled food or intravenous administration. These labelled fatty acids are either oxidized or incorporated into the lipid pools. This allows researchers to then assess synthesis of lipid depots, or fatty acid oxidation, and turnover. For example, studies in birds and mice provided food enriched with ^{13}C-palmitic acid measured the appearance of $^{13}CO_2$ in breath samples as a metric of whole-body lipid oxidation (Dick et al., 2020; Lyons et al., 2021). Furthermore, one research group used mice fed a ^{13}C-labelled fatty acid spiked diet over 6 months to quantify lipid turnover in a variety of tissues by sampling mice at specific time points and assessing tissue ^{13}C lipid content (Klievik et al., 2023a, 2023b). One disadvantage in these lipid turnover studies is that this often results in harvesting tissues and blood samples from multiple mice at a given timepoint, increasing the total number of animals in each experiment rather than longitudinal sampling from individual mice. Nonetheless, it provides a compelling approach to assessing lipid metabolism, with a large potential for future studies to also assess tissue lipid turnover under different metabolic conditions (i.e., intermittent fasting, exercise training, acclimation to different environmental temperatures, etc.; McCue and Welch, 2016). Furthermore, with these studies combined with indirect calorimetry, breath $^{13}CO_2$ can also provide estimations of dietary and/or circulating fatty acid systemic oxidation rates (McCue and Pollock, 2013; McCue and Welch, 2016; Carpentier, 2024). However, since metabolic pathways are both highly interconnected and compartmentalized, it can be difficult to isolate the contributions of specific tissues to whole-body energy expenditure if solely relying on breath $^{13}CO_2$. These techniques have been combined with arteriovenous sampling and biopsy techniques to estimate muscle, liver, cardiac, and white adipose tissue fatty acid uptake and release, albeit mainly in humans (Nielsen et al., 2004; Donnelly et al., 2005; Nelson et al., 2007). One research group was able to trace the fate and calculate flux of ^{13}C-labelled metabolites into a variety of tissues by infusing labelled tracers into conscious mice acclimated to either room temperature or to cold at 4 °C (Bornstein et al., 2023). Additionally, one recent preprint (Yuan et al., 2024, preprint) discusses how they integrate isotopic tracer infusion, isotopic gas analysis and mass spectrometry to quantify the oxidation and storage of 10 major circulating nutrients in fasting male mice. Most notably, they identified 3 non-esterified fatty acid species (palmitic, oleic, and linoleic) contributed 45% to total energy expenditure. It will be interesting to apply this technique for understanding metabolite contribution to mice undergoing different metabolic activities. It should also be noted that these studies can be resource-limiting, with restricted access to the necessary equipment for isotopic analysis, and financially limiting, as outsourcing analyses can be quite costly.

1.5. Case study: assessing circulating fatty acid distribution during exercise in deer mice

Assessing fatty acid oxidation during exercise is challenging in small animals such as mice. Popular tracer techniques, such as continuous tracer infusion, is particularly difficult in exercising mice. One alternative option to assess substrate use during exercise in mice is by administering an isotopic tracer immediately before exercise, and then sampling the animal immediately afterwards to quantify tissue isotopic signatures. For example, when the fatty acid tracer ^{14}C-2-bromopalmitic acid is administered as a bolus intravenously, it is transported and taken up by tissues with identical kinetics to palmitic acid; however, once it enters the cell, the bromine group prevents its entrance into the mitochondria, inhibiting its oxidation and thus trapping the radiolabelled fatty acid within the cell (similar to ^{18}FTHA as described above, Oakes et al., 1999). We have recently used ^{14}C-bromopalmitic acid to determine the fate of circulating fatty acids during a maximal cold challenge in deer mice (Lyons and McClelland, 2024).We found that during a maximal cold challenge, compared to individuals housed in thermoneutral conditions, there was a shift in fatty acid uptake with cold hypoxia acclimation in high-altitude deer mice to a greater accumulation in brown adipose tissue and less uptake into skeletal muscles. These tracer findings identified multiple tissues with significant fatty acid uptake and informed which tissues to focus on for further biochemical and molecular analyses, allowing us to gain a better comprehensive picture of the tissues being used during thermogenesis in deer mice.

Although we showed that this technique could be used to assess lipid metabolism at elevated metabolic rate during thermogenesis, it had not been used to examine other energetically taxing activities, such as exercise. Few studies have examined muscle recruitment or variation in fuel uptake with locomotory muscle in rodents (Armstrong et al., 1983; Schwane and Armstrong, 1983). Knowing which muscles are active or taking up fuels during exercise is important, as muscle recruitment patterns during progressively increasing running intensity are thought to help explain fuel use patterns (Roberts et al., 1996). Given our interest in exercise metabolism, we wanted to assess which muscles were taking up fatty acids at an exercise running intensity that elicits peak rates of lipid oxidation (~75% VO_2max) in deer mice (Lau et al., 2017). We adapted our previously used tracer technique methods (Lyons and McClelland, 2024) and running experiment protocols (Lau et al., 2017) to determine the fate of circulating fatty acids in a variety of muscles during running exercise. All procedures were approved by the McMaster University Animal Research Ethics Board in accordance with guidelines

set by the Canadian Council on Animal Care. In brief, deer mice (*Peromyscus maniculatus*) native to low altitude (Kearney, Nebraska, USA) were trained to be placed in a tail veiner restraint, receive a sham injection of saline intravenously via the tail vein, and familiarized to run on a mouse treadmill 3 times within 1 week. Only mice that had successfully completed the entire training period were used for experiments using ^{14}C-bromopalmitic acid. On the day of the experiment, mice (4 male and 2 female) were fasted for 4 h, given a bolus injection of ^{14}C-bromopalmitic acid intravenously via the tail vein, and then immediately placed on treadmill, running at a speed of 19.4 m/min to elicit ~75% of running VO_2max for 15 min (Lau et al., 2017). After 15 min of running, mice were immediately sacrificed using isoflurane, and tissues were quickly dissected and snap frozen in liquid nitrogen until further analysis. Tissues were later digested and counted for ^{14}C activity as described previously (Lyons and McClelland, 2024), indicating tissue fatty acid uptake.

We sampled 20 tissues to describe the relative fatty acid uptake distribution into 16 muscles (63% of total activity), 2 brown adipose (27.5%) and 2 white adipose tissue (~9.5%) depots during submaximal exercise in deer mice (Fig. 1). Within the muscles sampled, the upper hindlimb muscles (bicep femoris, semitendinosus, rectus femoris) and central core muscles (erector spinae, gluteus) had greater relative uptake of fatty acids compared to forelimb (triceps and biceps) and lower hindlimb muscles (tibialis anterior, gastrocnemius, extensor digitorum longus). As well, oxidative muscle (red gastrocnemius) tended to have relatively greater fatty acid uptake compared to more glycolytic muscle (white gastrocnemius; $p = 0.07$). Interestingly, the values of fatty acid uptake in the hindlimb muscles during exercise are relatively greater than those observed during thermogenesis (Lyons and McClelland, 2024), supporting the idea that muscles involved in running are receiving more blood flow and taking up more fatty acids than shivering muscle.

However, in keeping with the theme of this commentary, using ^{14}C-2-bromopalmitic acid in exercising mice does come with its limitations. Ideally, cannulation of the carotid artery and jugular vein to infuse tracers and collect blood would enable the calculation of fatty acid uptake rate into working tissues. However, given the small size of the mice and nature of our running protocol, it was not feasible to conduct surgery and safely sample adequate volumes of blood sequentially over the course of the experiment. Furthermore, the timing of tracer administration and tissue sampling is crucial to interpretation of results. For example, giving the tracer immediately before the run may reflect what happens to blood flow and fatty acid uptake in transition from rest to exercise, while giving the tracer immediately after exercise might reflect blood flow and fatty acid uptake during post-exercise recovery. Lastly, given the nature of the ^{14}C-bromopalmitic acid tracer, it becomes trapped within the tissue, but it is unclear the proportion that is destined for oxidation versus esterified into intramuscular triglyceride stores. Submaximal running at ~75% VO_2max likely represents an intensity where whole-animal lipid oxidation is highest during exercise. Therefore, most of the fatty acids taken up into the muscle are probably directed towards oxidation; however, the metabolic fate of the circulatory fatty acids in running deer mice is unknown. Using an additional radiolabelled fatty acid, such as ^{3}H-palmitic acid, would provide a better idea on how much of the taken up fatty acid is being oxidized, as measured by $^{3}H_2O$ found in either the tissue or plasma, or the remaining ^{3}H activity found in the tissue's lipid phase (Schönke et al., 2018).

2. Conclusions

In summary, isotopic tracers represent a powerful tool for assessing whole-animal fuel utilization and tissue-specific metabolism. By providing quantitative data on substrate fluxes and metabolic pathways, these tracers offer insights into the complex interplay between metabolism and physiological function. While these techniques are powerful and informative, they can have frustrating limitations that challenge

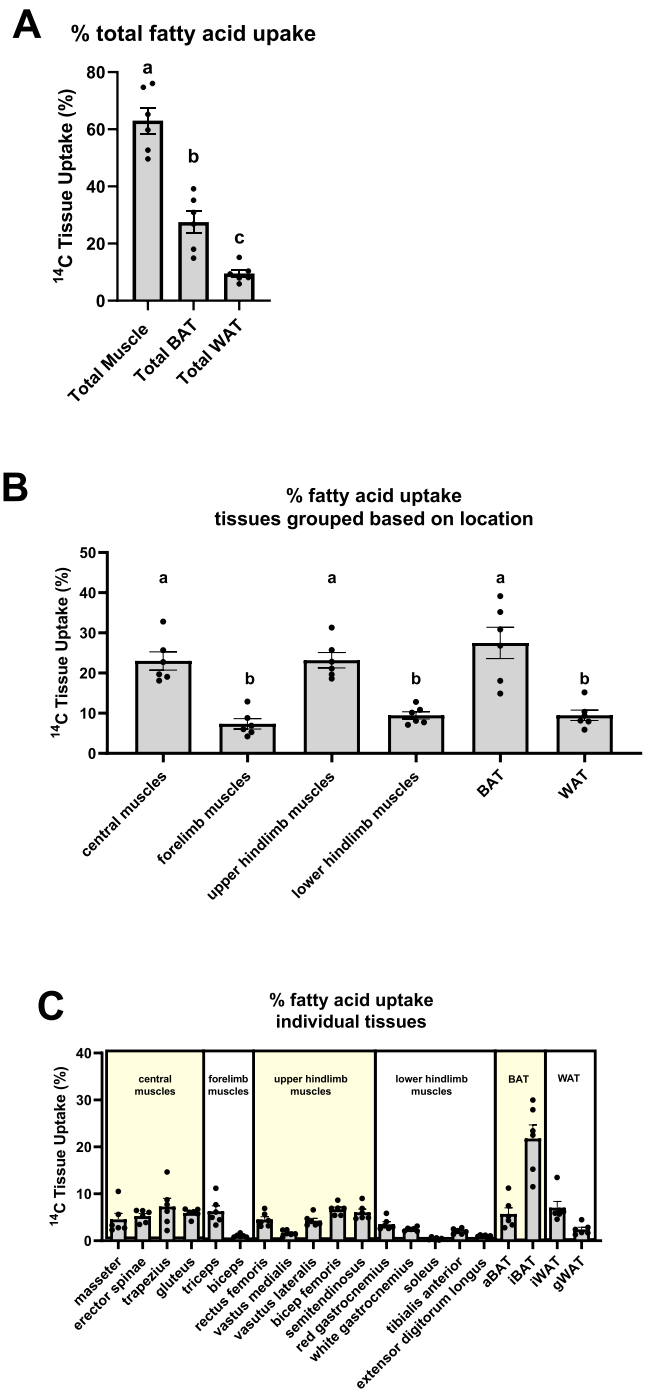

Fig. 1. Percent distribution of ^{14}C-2-bromopalmitic acid in the tissues of lowland deer mice (*Peromyscus maniculatus*) run at submaximal exercise (75% of running $V\dot{O}_2$max) for 15 min. A: % ^{14}C-bromopalmitic acid uptake in total muscle, white and brown adipose tissue; B: % ^{14}C-bromopalmitic acid uptake in tissue groups based on anatomical location. C: % ^{14}C-bromopalmitic acid uptake in each individual tissue. $N = 4$ male and 2 female mice. 1-way ANOVA with a Tukey's multiple comparisons test; different letters denote significance differences ($p < 0.05$). Data presented as mean ± SEM. aBAT, auxiliary brown adipose tissue; EDL, extensor digitorum longus; iBAT, interscapular brown adipose tissue; iWAT, interscapular white adipose tissue; gWAT, gonadal white adipose tissue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

researchers and limit their widespread use, particularly in smaller animals at anything beyond the resting condition. These limitations can complicate the ease of tracer-based experiments when assessing fuel use contributions to whole-body energy expenditure, thus limiting our understanding of the relationship between tissue substrate metabolism and whole-body energy expenditure. To overcome these limitations to assess substrate use during conditions such as exercise in mice, creative solutions to these problems involve using techniques and experimental designs to circumvent techniques that may be barriers for most researchers working with mice (i.e., vessel cannulation). For example, using bolus intravenous injections of non-oxidizable isotopic tracers (i.e., ^{14}C-bromopalmitic acid, ^{18}FTHA) in resting versus exercised mice can provide information regarding differences in tissue blood flow and substrate uptake. Or another example, using stable isotopic tracers in the diet to assess tissue substrate turnover in resting and exercising mice, in combination with indirect calorimetry to tease apart substrate oxidation vs storage. The use of tracer studies provides a wealth of information and may identify key tissues of interest for in-depth biochemical and/or molecular analyses. Overall, isotopic tracer research has significantly advanced our understanding of substrate use metabolism. Given how important mice are to current research, we need to keep advancing techniques and experimental designs that allow us to further our understanding of energy expenditure in smaller animals under different metabolic conditions.

Funding

This work was funded by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant and a NSERC Discovery Accelerator Supplement (G.B.M.). S.A.L was supported by a NSERC Canada Graduate Scholarship.

CRediT authorship contribution statement

Sulayman A. Lyons: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Grant B. McClelland:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank Lauren Dessureault for technical assistance with running mice on treadmills.

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