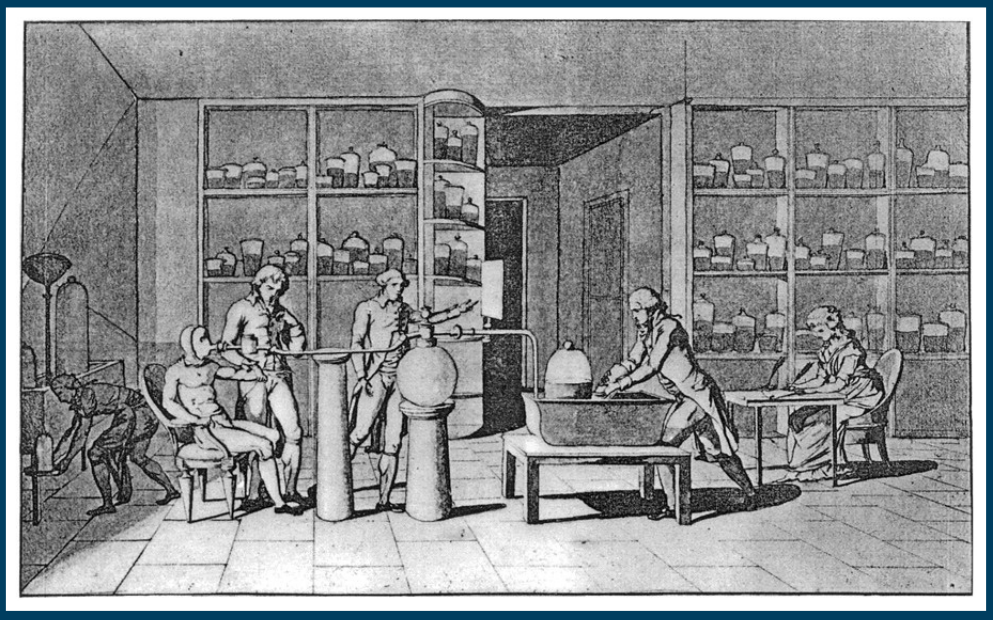
frontiers

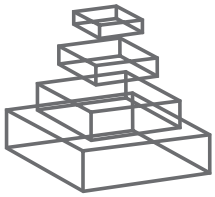
RESEARCH TOPICS



ENERGY METABOLISM

Topic Editor
Patrick C. Even

frontiers in PHYSIOLOGY



frontiers

ISSN 1664-8714
ISBN 978-2-88919-308-0
DOI 10.3389/978-2-88919-308-0

ABOUT FRONTIERS

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

FRONTIERS JOURNAL SERIES

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing.
All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

DEDICATION TO QUALITY

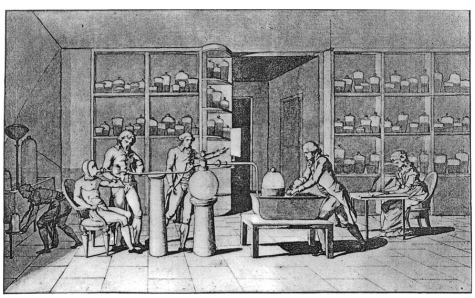
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

WHAT ARE FRONTIERS RESEARCH TOPICS?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area!
Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

ENERGY METABOLISM

Topic Editor:
Patrick C. Even, AgroParisTech, France



Kenneth J. Carpenter, "A Short History of Nutritional Science: Part 1 (1785–1885)", The Journal of Nutrition, March 1, 2003, accessed July 2, 2014, http://jn.nutrition.org/content/133/3/638.full

Energy metabolism is central to life and altered energy expenditure (EE) is often cited as a central mechanism responsible for development of the obese phenotype. Resting EE, EE of physical activity, cold induced thermogenesis and thermic effect of feeding add to produce total EE but can also affect each other. It is thus very important that each component be well measured.

Measuring energy expenditure by indirect calorimetry is extremely simple in theory but the practice if far more difficult. Taking into account temperature in small sized animals, measuring accurately the effect of activity on EE, correcting EE for body size body composition, age sex etc… add difficulties in producing reliable data.

The goal of this Research Topic was to call for the practical experience of main investigators trained to practice calorimetry in order to get their feedback and the way they deal with the various and specific problems of humans and animal calorimetry. The goal is to share the questions/solutions experienced by the contributors to inititate a "guide of the good practices" that can be periodically updated and used by all those who are and will be interested in measuring energy metabolism from the 20g mouse to the human and large farm animals.

Table of Contents

frontiers in
PHYSIOLOGY

EDITORIAL
published: 17 October 2013
doi: 10.3389/fphys.2013.00301

Body size, spontaneous activity and thermogenesis effects on energy expenditure: an introduction to a topic on energy metabolism

Patrick C. Even*

UMR914 Nutrition Physiology and Ingestive Behavior, AgroParisTech, INRA, Paris, France
**Correspondence: even@agroparistech.fr*

Edited by:
Geoffrey A. Head, BakerIDI Heart and Diabetes Institute, Australia

Keywords: energy expenditure, indirect calorimetry, body size, body composition, physical activity, brown adipose tissue, metabolic phenotyping, thermogenesis

Life is sustained by the extraction of energy from nutrients. The mechanism is oxidation of the energy-containing macronutrients in food: carbohydrates, lipids and proteins. This result in rates of oxygen consumption and carbon dioxide release closely proportional to the energy extracted from the nutrients. So in effect, measuring respiratory exchanges is measuring life itself.

The measurement of whole body energy expenditure (EE) and substrate utilization by continuous recording of oxygen consumption, carbon dioxide production and when required nitrogen and methane excretion is based on techniques that matured early in the previous century thanks to pioneering work led by researchers such as Rubner, Lusk and Benedict. For those interested in an historical perspective on the concepts of respiration and calorimetry, I strongly suggest reading the richly illustrated review of Frankenfield (2010).

Indirect calorimetry reveals the overall integration of the metabolic pathways controlling energy fluxes and partitioning, and informs if an observed or provoked alteration at the cellular or organ level bears significant consequences at the whole body level. This technique, although appearing simple in its principles, is in fact very sensitive to methodological and conceptual errors, and so requires great care to be correctly used and interpreted. It also raises different problems when applied to humans or to small laboratory rodents. Many technical reviews have been published on this subject, discussing in both animals and humans the apparatus design (Jensen et al., 2001; Kaiyala and Ramsay, 2010; Melanson et al., 2010; Even and Nadkarni, 2012), data processing (Arch et al., 2006; Compher et al., 2006; Schoffelen and Westerterp, 2008; Tschop et al., 2012) and possible limitations of the technique (Walsberg and Hoffman, 2005). Contributors to this topic have focused on three main components of EE susceptible to affect measurements and interpretation of the data; body size, spontaneous activity and thermogenesis.

Whole body EE must be properly adjusted for body size and composition to avoid incorrect interpretations. This is because tissues and organs have different specific metabolic rates, and more globally fat free mass has a larger influence on EE than fat mass (Elia, 1992). This has been extensively discussed by John Arch (Arch and Trayhurn, 2013), plus Anja Bosy-Westphal has reviewed the main strategies used in humans to adjust EE to body size, proposing a new approach to improve adjustment of EE between subjects with wide differences in percent fat mass (Bosy-Westphal et al., 2013). In his review on measuring energy metabolism in the mouse, John Speakman considered the various attempts made to deal with this question in the mouse model (Speakman, 2013).

One main source of variability in total EE is related to the amount of spontaneous activity. It has been a challenge for years to precisely measure it and then compute the consequent energy expenditure. The technical problems may be very different depending on the subject (human vs. animal) and experimental conditions (chambers vs. field measurements). On this topic, Dr Westerterp discussed methods for measurement, determinants and effects of physical activity in humans. He also focused on the interest of the doubly labeled water technique as a field indirect calorimetry method to assess physical activity in humans (Westerterp, 2013). Dr Sarafian (from the laboratory of Dr Dulloo) described a new approach to perform standardized tests for assessing human variability in the energy cost of low-intensity isometric work that is comparable to daily life activities (Sarafian et al., 2013). Etienne Labussière described the method used in his laboratory to deal with this question in large farm animals such as white pigs (Labussiere et al., 2013), and Jan B van Klinken described the most advanced procedures for rats and mice and compared their robustness in relation to the frequency of data acquisition and quality of the activity signal (van Klinken et al., 2013). In his review, John Speakman developed an extensive section on physical activity in which he discussed the various issues related to this question (including treadmill running) and surveyed the most significant published works (Speakman, 2013).

Brown adipose tissue (BAT) is essential in small rodents to maintain body temperature. Since thermoneutrality occurs at 28–32°C, BAT is already very active at the 21°C ambient temperature in most animal facilities. This can increase resting EE in mice up to 2–3 fold when singly housed (Selman et al., 2001) (see also Speakman, 2013) or by 60% when housed in groups with isolative bedding (Cannon and Nedergaard, 2009). In the rat, decreasing temperature from 30 to 20°C increases resting EE by 50% (Evans et al., 2005). Understanding the effect of BAT is thus essential for mouse but also for rat metabolic phenotyping. In humans, until recently it was considered that BAT disappeared rapidly after birth. However, significant amounts of active BAT in humans have been revealed by positron emission tomography (Nedergaard et al., 2007). Acute exposure to cold

stimulated and propranolol treatment inhibited activity of these depots (Nedergaard et al., 2010). Thus in a context where no safe and effective drugs are available to treat obesity, this observation has motivated researchers into considering the possibility that stimulating BAT might be effective in treating obesity and its associated metabolic disorders (Bartelt et al., 2011). Sam Virtue has discussed the practical considerations of methods for analyzing BAT tissue function in rodents, including the use of indirect calorimetry and other more simple measurements such as pair feeding, BAT lipid content and protein markers (Virtue and Vidal-Puig, 2013), and Jonathan Arch has discussed the detection of thermogenesis in rodents in response to anti-obesity drugs and genetic modification. He asserts that a proper analysis of the thermogenic response to genetic modifications or pharmacological compounds is essential to decide whether it is worth seeking drugs potentially useful for obesity treatment (Arch and Trayhurn, 2013). Both also reminded us that thermogenesis may be stimulated outside BAT, and drew our attention to the risk of confounding effects on thermogenesis with those on locomotor activity or body composition.

There is much more in each of the articles on this topic than can be commented on in this short introduction. I hope that the readers will go through the articles where they will find a lot of information to better master the possibilities and understand the limitations of measuring EE in humans and animals.

ACKNOWLEDGMENTS

Patrick C. Even is funded by CNRS.

REFERENCES

Arch, J. R., and Trayhurn, P. (2013). Detection of thermogenesis in rodents in response to anti-obesity drugs and genetic modification. *Front. Physiol.* 4:64. doi: 10.3389/fphys.2013.00064

Arch, J. R., Hislop, D., Wang, S. J., and Speakman, J. R. (2006). Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals. *Int. J. Obes.* 30, 1322–1331. doi: 10.1038/sj.ijo.0803280

Bartelt, A., Bruns, O. T., Reimer, R., Hohenberg, H., Ittrich, H., Peldschus, K., et al. (2011). Brown adipose tissue activity controls triglyceride clearance. *Nat. Med.* 17, 200–205. doi: 10.1038/nm.2297

Bosy-Westphal, A., Braun, W., Schautz, B., and Muller, M. J. (2013). Issues in characterizing resting energy expenditure in obesity and after weight loss. *Front. Physiol.* 4:47. doi: 10.3389/fphys.2013.00047

Cannon, B., and Nedergaard, J. (2009). Thermogenesis challenges the adipostat hypothesis for body-weight control. *Proc. Nutr. Soc.* 68, 401–407. doi: 10.1017/S0029665109990255

Compher, C., Frankenfield, D., Keim, N., and Roth-Yousey, L. (2006). Best practice methods to apply to measurement of resting metabolic rate in adults: a systematic review. *J. Am. Diet. Assoc.* 106, 881–903. doi: 10.1016/j.jada.2006.02.009

Elia, M. (1992). "Organ and tissue contribution to metabolic rate," in *Enrgy Metabolism: Tissue Determinants and Cellular Corollaries*, eds J. M. Kinney and H. N. Tuckey (NewYork, NY: Press), 61–77.

Evans, S. A., Parsons, A. D., and Overton, J. M. (2005). Homeostatic responses to caloric restriction: influence of background metabolic rate. *J. Appl. Physiol.* 99, 1336–1342. doi: 10.1152/japplphysiol.01380.2004

Even, P. C., and Nadkarni, N. A. (2012). Indirect calorimetry in laboratory mice and rats: principles, practical considerations, interpretation and perspectives. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 303, R459–R476. doi: 10.1152/ajpregu.00137.2012

Frankenfield, D. C. (2010). On heat, respiration, and calorimetry. *Nutrition* 26, 939–950. doi: 10.1016/j.nut.2010.01.002

Jensen, D. R., Gayles, E. C., Ammon, S., Phillips, R., and Eckel, R. H. (2001). A self-correcting indirect calorimeter system for the measurement of energy balance in small animals. *J. Appl. Physiol.* 90, 912–918.

Kaiyala, K. J., and Ramsay, D. S. (2010). Direct animal calorimetry, the underused gold standard for quantifying the fire of life. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 158, 252–264. doi: 10.1016/j.cbpa.2010.04.013

Labussiere, E., Dubois, S., van Milgen, J., and Noblet, J. (2013). Partitioning of heat production in growing pigs as a tool to improve the determination of efficiency of energy utilization. *Front. Physiol.* 4:146. doi: 10.3389/fphys.2013.00146

Melanson, E. L., Ingebrigtsen, J. P., Bergouignan, A., Ohkawara, K., Kohrt, W. M., and Lighton, J. R. (2010). A new approach for flow-through respirometry measurements in humans. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 298, R1571–R1579. doi: 10.1152/ajpregu.00055.2010

Nedergaard, J., Bengtsson, T., and Cannon, B. (2007). Unexpected evidence for active brown adipose tissue in adult humans. *Am. J. Physiol. Endocrinol. Metab.* 293, E444–E452. doi: 10.1152/ajpendo.00691.2006

Nedergaard, J., Bengtsson, T., and Cannon, B. (2010). Three years with adult human brown adipose tissue. *Ann. N. Y. Acad. Sci.* 1212, E20–E36. doi: 10.1111/j.1749-6632.2010.05905.x

Sarafian, D., Miles-Chan, J. L., Yepuri, G., Montani, J. P., Schutz, Y., and Dulloo, A. G. (2013). A standardized approach to study human variability in isometric thermogenesis during low-intensity physical activity. *Front. Physiol.* 4:155. doi: 10.3389/fphys.2013.00155

Schoffelen, P. F., and Westerterp, K. R. (2008). Intra-individual variability and adaptation of overnight- and sleeping metabolic rate. *Physiol. Behav.* 94, 158–163. doi: 10.1016/j.physbeh.2007.12.013

Selman, C., Korhonen, T. K., Bunger, L., Hill, W. G., and Speakman, J. R. (2001). Thermoregulatory responses of two mouse *Mus musculus* strains selectively bred for high and low food intake. *J. Comp. Physiol.* 171, 661–668. doi: 10.1007/s003600100217

Speakman, J. R. (2013). Measuring energy metabolism in the mouse - theoretical, practical, and analytical considerations. *Front. Physiol.* 4:34. doi: 10.3389/fphys.2013.00034

Tschop, M. H., Speakman, J. R., Arch, J. R., Auwerx, J., Bruning, J. C., Chan, L., et al. (2012). A guide to analysis of mouse energy metabolism. *Nat. Methods* 9, 57–63. doi: 10.1038/nmeth.1806

van Klinken, J. B., van den Berg, S. A., and van Dijk, K. W. (2013). Practical aspects of estimating energy components in rodents. *Front. Physiol.* 4:94. doi: 10.3389/fphys.2013.00094

Virtue, S., and Vidal-Puig, A. (2013). Assessment of brown adipose tissue function. *Front. Physiol.* 4:128. doi: 10.3389/fphys.2013.00128

Walsberg, G. E., and Hoffman, T. C. (2005). Direct calorimetry reveals large errors in respirometric estimates of energy expenditure. *J. Exp. Biol.* 208, 1035–1043. doi: 10.1242/jeb.01477

Westerterp, K. R. (2013). Physical activity and physical activity induced energy expenditure in humans: measurement, determinants, and effects. *Front. Physiol.* 4:90. doi: 10.3389/fphys.2013.00090

Received: 04 September 2013; accepted: 01 October 2013; published online: 17 October 2013.

Citation: Even PC (2013) Body size, spontaneous activity and thermogenesis effects on energy expenditure: an introduction to a topic on energy metabolism. Front. Physiol. ***4****:301. doi: 10.3389/fphys.2013.00301*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 14 March 2013
doi: 10.3389/fphys.2013.00034

Measuring energy metabolism in the mouse – theoretical, practical, and analytical considerations

John R. Speakman*[1,2]

[1] *Key State Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China*
[2] *Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen, Scotland, UK*

Edited by:
Patrick C. Even, AgroParisTech, France

Reviewed by:
Jon Arch, University of Buckingham, UK
Jan Bert V. Klinken, Leiden University Medical Centre, Netherlands

****Correspondence:***
John R. Speakman, Key State Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, 1 West Beichen Road, Chaoyang, Beijing 100100, China.
e-mail: j.speakman@abdn.ac.uk

The mouse is one of the most important model organisms for understanding human genetic function and disease. This includes characterization of the factors that influence energy expenditure and dysregulation of energy balance leading to obesity and its sequelae. Measuring energy metabolism in the mouse presents a challenge because the animals are small, and in this respect it presents similar challenges to measuring energy demands in many other species of small mammal. This paper considers some theoretical, practical, and analytical considerations to be considered when measuring energy expenditure in mice. Theoretically total daily energy expenditure is comprised of several different components: basal or resting expenditure, physical activity, thermoregulation, and the thermic effect of food. Energy expenditure in mice is normally measured using open flow indirect calorimetry apparatus. Two types of system are available – one of which involves a single small Spartan chamber linked to a single analyzer, which is ideal for measuring the individual components of energy demand. The other type of system involves a large chamber which mimics the home cage environment and is generally configured with several chambers/analyzer. These latter systems are ideal for measuring total daily energy expenditure but at present do not allow accurate decomposition of the total expenditure into its components. The greatest analytical challenge for mouse expenditure data is how to account for body size differences between individuals. This has been a matter of some discussion for at least 120 years. The statistically most appropriate approach is to use analysis of covariance with individual aspects of body composition as independent predictors.

Keywords: energy metabolism, indirect calorimetry, mouse models, energy balance, obesity, physical activity, basal metabolic rate, energy expenditure

OVERVIEW

The mouse is probably the most important species as a model for the study of human diseases and disorders. Despite millions of years of evolutionary divergence the mouse has extremely close synteny of its genome with the human (Peltonen and McKusick, 2001), and physiologically, being a mammal and an endotherm, it shares many features of human metabolism not found in the other animal models such as ectothermic invertebrates like *Drosophila melanogaster* and *Caenorhabditis elegans*. The rat is also a mammalian endotherm that shares much of its genome and physiology with the human. What sets the mouse apart, however, is the technological capability to manipulate its genome to generate animals with global and tissue specific knock-out and transgenic models. This gives us phenomenal capabilities to explore the relationships between individual and multiple genes and their phenotypic consequences. Ascertaining the functions of the 30,000 or so genes in the human genome will be facilitated enormously by the study of the mouse in the coming decades.

Part of this effort will be to understand the impact that individual genes have on energy metabolism, and their consequences for disorders such as obesity (Speakman et al., 2008; Hall et al., 2012). This paper concerns theoretical and practical considerations for measuring the energy metabolism of the mouse. It also addresses the issue of how to analyze the resulting data and some of the pitfalls in this analysis. These considerations apply more generally to other small mammals in the same size range as mice (i.e., <100 g). I will therefore also draw on some examples in the literature of studies on such animals. Several other publications contain useful information on similar issues that are directly pertinent to the measurement of energy metabolism in the mouse and the reader may also wish to consult these, in particular the papers by Weir (1949), Kleiber (1961), Ferrannini (1988), Simonson and deFronzo (1990), Bursztein et al. (1989), Elia and Livesey (1992), Even et al. (1994), Arch et al. (2006), Lighton (2008), Tschoep et al. (2012), Even and Nadkarni (2012), Speakman et al. (2013).

THEORETICAL CONSIDERATIONS

DAILY ENERGY EXPENDITURE AND ITS COMPONENTS

The total daily expenditure of energy (variously called TEE, TDEE, or DEE) can be partitioned into different components. These normally include the energy spent on basal metabolism, the thermic effect of food (the increase in energy expenditure following food intake which is also called the heat increment of feeding or the specific dynamic action), the energy spent on thermoregulation and the energy spent on physical activity. These components are often presented as a tower block shaded in different ways to reflect

the different components and their relative sizes. However, a fundamental assumption being made in this type of diagram is that the components, as defined, are independent and additive. This may not be the case. Heat generated by activity or feeding, for example, may substitute for the costs of thermoregulation in some circumstances (Zerba and Walsberg, 1992; Bruinzeel and Piersma, 1998; Bech and Praesteng, 2004; Humphries and Careau, 2011; Virtue et al., 2012). Researchers may be interested in the impact of a given manipulation on the total daily energy expenditure and/or the components of expenditure. Ability to accurately measure the total daily expenditure or the different components depends on the type of equipment available.

INDIRECT VERSUS DIRECT CALORIMETRY FOR MOUSE MEASUREMENTS

There are two fundamentally different ways to measure energy metabolism. The end product of all metabolic activity is either heat or work. Since work also ultimately appears as heat, one way is to measure the heat produced directly by the animal. This is called direct calorimetry. Direct calorimetry was popular in the first half of the last century but it fell out of favor because it is difficult to use, mostly because measuring small amounts of heat is technically challenging. Moreover, it makes a critical assumption that no heat is stored in the animals' body during the measurement period. The error induced by this assumption can be quite large. Imagine a 30 g mouse is in a direct calorimeter and it is expending 0.35 W. If its body temperature was to rise by 1°C over the course of an hour in the chamber then it would have stored 125.5 J of heat (assuming the specific heat capacity of body tissue is about the same as that for water). This would be equivalent to 0.035 W (125.5/3,600), or 10% of the metabolic rate. So the actual measured heat production would be 10% too low. Equally if it cooled down by 1°C then the heat production estimate would be 10% higher than the actual metabolism, by virtue of the released body heat. Such changes in body temperature may routinely occur during measurements of energy expenditure (**Figure 1**).

The alternative is to not measure the heat directly but rather measure components of the metabolic process that generate the heat, and hence infer its production indirectly. This has become known as indirect calorimetry, or, because respiratory gases are used, respirometry.

The overall equation for the metabolism of glucose for example is

$$C_6H_{12}O_6 + 6\,(H_2O) + 6\,(O_2) \rightarrow 12\,(H_2O) + 6\,(CO_2) + 2820\,\text{kJ} \quad (1)$$

Hence we know from this equation that for every 6 mol of oxygen consumed or CO_2 produced that the animal has used 2,820 kJ of energy. Fortunately measuring oxygen and CO_2 gases can be performed with great accuracy: much better accuracy than for the small amounts of heat involved. Moreover, these compounds are not normally stored in the body to any great extent – unlike heat. The only downside of this approach is that animals do not always metabolize glucose, and when they change to burning other substrates the equation changes. However the equation changes in a systematic way depending on the substrate being used. This can be diagnosed from the measured ratio of oxygen consumption to

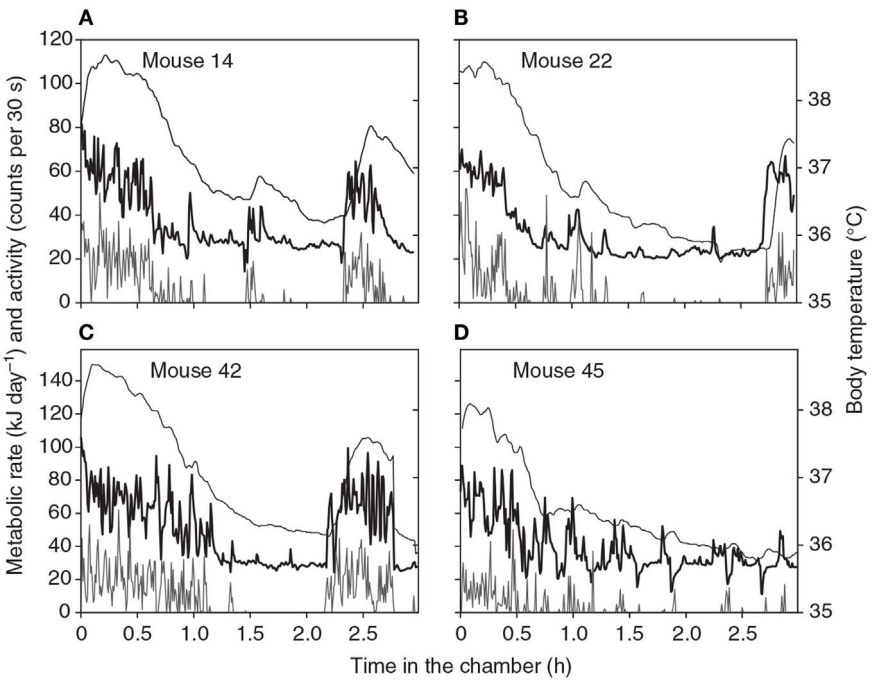


FIGURE 1 | Patterns of energy expenditure (thick black line: kJ/day) body temperature (thin black line: °C) and physical activity (gray line: "counts"/30 s) during four 3-h long respirometry measurements of four different individual mice. Data are from a single chamber-single analyzer system with a small 1.25 L chamber. Measurements made every 30 s (from Duarte et al., 2010).

CO_2 production (called the respiratory exchange ratio: RER). The actual substrate oxidation at the tissue level is called the respiratory quotient (RQ). RQ is reflected in the RER but because of lags in the body they are not directly equivalent over short timescales. If we can work out how much nitrogen has been produced via the urine to calculate protein oxidation, then we can work out the other substrate oxidations from the RER (Weir, 1949), and very accurately calculate the energy expenditure. In fact, not correcting for differences in protein oxidation induces only a small error, unless protein oxidation exceeds 15% (Even and Nadkarni, 2012) and most people ignore this effect, using only the oxygen consumption combined with the estimated RQ from simultaneous measurements of CO_2 production. The result is in theory accurate to within 1–2% of the true energy expenditure (Weir, 1949; Ferrannini, 1988), but see Walsberg and Hoffman (2005) for data showing that in practice discrepancies can be much higher. An argument has been made that assumptions underlying indirect calorimetry methodology remain untested in genetically modified mice and using both direct and indirect calorimetry in tandem may be a useful way forwards (Kaiyala and Ramsay, 2011).

The first systems to measure oxygen consumption of mice were closed systems (Davis and van Dyke, 1932, 1933). The animals were placed in a sealed chamber with a chemical that absorbs CO_2 (generally calcium carbonate or soda lime) and the resultant consumption of oxygen altered the internal pressure which could be measured using a manometer. These systems, however, have two problems. First, it is only possible with this system to measure oxygen consumption. In theory it is feasible to measure the RQ by omitting the CO_2 absorber and measuring volume changes, but this is very inaccurate as the volume changes are small and confounded by water vapor changes. Second, the mouse inside the chamber can perform lots of other behaviors as well as resting and it is difficult to separate out these effects. A method to try and eliminate the major source of this artifact was developed in the 1930s and involved simply waiting until the mouse settled down before fully sealing the chamber (Davis and van Dyke, 1932), but if it wakes up again afterward the measure is compromised.

Many measurements have been made using such systems and, despite being unable to accurately diagnose the substrate being oxidized, because of no RQ estimate, the data generated are quite good. The reason for this is that the error in converting from oxygen consumption to energy demands in the absence of a known RQ is relatively small. Nevertheless, the inability to adequately separate resting and non-resting behavior, and the construction of gas analyzers that could measure gas concentrations continuously, led eventually to the development of open flow respirometry systems, and these currently dominate the field. Very few people still use closed systems or direct calorimetry. In an open flow system the chamber is connected into a continuous flow of gas. Hence the "sealed" chamber has an incurrent and excurrent gas flow. Typically the gas will be drawn from an atmospheric source by a pump and then dried using silica gel. The gas flow will then be measured and regulated by a mass flow controller before entering the chamber where the animal is placed. The chamber will also have an outflow, downstream of which the gases will be dried again to remove any moisture introduced by the animal, and then passed into the gas analyzer where O_2, and/or CO_2 concentrations will be measured. Sophisticated systems may have a parallel stream that does not contain an animal and passes into a second channel of the analyzer to provide a constant reference point for atmospheric gas levels. These dual flow analyzer systems provide the most accurate estimates of oxygen consumption and CO_2 production. However, if there is only a single analyzer channel then the background oxygen and CO_2 levels are generally imputed from the start and end concentrations, by performing a "drift" correction. Generally when the measurement period is short ($<$3 to 4 h) then making a single start to end drift correction is adequate. However, if the measurement is longer it is often necessary to interrupt the measurement to obtain a background estimate. This is not as simple as it may first appear because the chamber where the animal resides must continue to be ventilated at exactly the same rate while the background is being measured.

This level of flow control only became technologically possible with the advent of mass flow controllers that allowed a regulated fixed flow of gas to enter the chamber independent of the pump supplying the gas. Whether the reference measurement is obtained directly, or imputed from single or multiple drift corrections, the measured or calculated difference in gas concentrations between the reference and the sample channels provides an estimate of the O_2 and CO_2 concentration changes produced by the animal in the chamber. The maths for calculating the oxygen consumption and CO_2 production in such systems were worked out many years ago and summarized by Weir (1949), see also Even et al. (1994), Arch et al. (2006), Lighton (2008).

A point to note here is the importance of drawing the incurrent gas stream from outside the room where the measurements are being made, preferably a completely fresh airstream. This is because the background CO_2 and O_2 contents of the room atmosphere can be significantly impacted by the presence of the researcher or other staff in the room. This may compromise the assumption of linear drift. In addition it may not be obvious but the position of the pump has a bearing on the reliability of the system and influences exactly where the flow rate should be regulated and measured. As a general rule the flow rate should be regulated and measured immediately adjacent to the pump. If the pump is placed upstream of the chamber the system runs under slight positive pressure, and if it is placed downstream it is under slight negative pressure. This influences what happens if there is a slight leak in the chamber. In a system running under positive pressure (pump in incurrent stream) some gas will leak out of the chamber. This will be at the same concentration as that exiting down the excurrent tube to the analyzer, so will not influence the analyzer reading, but clearly if you pump xx ml into the chamber but $<$xx ml goes down the excurrent tube, if you monitor the flow in the excurrent tube rather than adjacent to the pump, in the incurrent tube, you will have an error in the flowrate equal to the magnitude of the leak. Similarly if the system runs under negative pressure (pump in excurrent flow) then if there is a slight leak in the chamber, atmospheric gases will be drawn in via the leak as well as via the incurrent tube. Again if you draw xx ml out of the chamber via the excurrent tube but $<$xx is coming in via the incurrent tube you will have an error in the flowrate the magnitude of the leak if you monitor and regulate the flow via the incurrent stream, rather than adjacent to the pump in the excurrent flow.

The exact calculation is also dependent on whether the flow is measured upstream or downstream of the chamber (for details refer to Weir, 1949; Ferrannini, 1988; Even et al., 1994; Arch et al., 2006).

If both CO_2 and O_2 are measured then the resultant oxygen consumption can be converted into an energy expenditure measurement using the inferred substrate utilization (RQ) from the measured RER, assuming negligible protein oxidation has occurred. However, if only oxygen (or only CO_2) is measured then it is necessary to assume an RQ value to derive the energy expenditure. Unless there is good reason to expect the animal is metabolizing exclusively fat, or a known diet with a given composition (food quotient) then generally the unknown RQ is assumed to be 0.8 or 0.85, as values between 0.7 (pure fat oxidation) and 1.0 (pure carbohydrate oxidation) minimize the error in the assumption. It should be noted that the potential error for converting oxygen consumption to energy expenditure is much smaller than the potential error converting CO_2 production to energy expenditure, when the actual RQ is unknown Hence, if sufficient resources are available only to purchase either an O_2 analyzer or a CO_2 analyzer, one is better to buy the O_2 analyzer. Moreover, if you read literature based only on CO_2 estimates then it is good to be aware of the potential errors involved in the extrapolation to energy when RQ is unknown. This also applies to the doubly labeled water and labeled bicarbonate methods (below) which measure only CO_2 production.

An issue to be considered here is if one has only an O_2 analyzer is it better to also absorb the CO_2 as well as the water vapor from the stream of gas exiting the chamber (Arch et al., 2006). The reasoning behind this is that the CO_2 dilutes the oxygen concentration to some unknown extent and this can introduce an error into the estimated VO_2. If you are interested in measuring oxygen consumption then to obtain the most accurate estimate it is best to absorb both the CO_2 and the water in the excurrent stream. The equations to use with this type of configuration were established over a century ago by Haldane (1912) and are reiterated in detail in Weir (1949), Even et al. (1994), and Arch et al. (2006). Perhaps surprisingly if you are interested in energy expenditure rather than oxygen consumption then this configuration does not give the most accurate result (Koteja, 1996; Speakman, 2000). The reason is that there are actually two assumptions and errors being made in the whole process of going from oxygen concentration measurements to energy expenditure. The first assumption, if the CO_2 is not absorbed, is the extent of the dilution due to the unknown amount of CO_2 present. The error resulting from this assumption depends on what the actual RQ is relative to the RQ that is assumed. However, there is a second assumption when converting the oxygen consumption into energy expenditure, and that is what the oxycalorific equivalent of the consumed oxygen is. There is consequently also an error that depends on the difference between the assumed and the actual RQ. These two errors almost completely cancel each other out (Koteja, 1996). The net result is that if you absorb the CO_2 you get a more accurate estimate of O_2 consumption, but a worse estimate of the energy expenditure because you have removed one of the two errors that cancel each other out. So the message is clear. If your primary interest is energy and not oxygen then do not absorb the CO_2 from the excurrent stream of the respirometry chamber.

SINGLE CHANNEL OR MULTICHANNEL SYSTEMS

In a single channel system, a single chamber is positioned in a gas flow that goes into a single analyzer. There may or may not be a second channel used as a reference channel but the main distinguishing point of these systems is that the animal in question is measured for the entire time it is in the chamber. The key problem with such systems is that unless you have lots of them (which is expensive) then measuring multiple animals is a slow process. If for example one was interested in characterizing mouse basal energy demands for which a 3–4 h measurement is typical it would be difficult to get more than one measurement into a standard working day (if prior starvation time is taken into account – see below), making the normal throughput about five animals/week. The invention of mass flow controllers however meant that several chambers could be simultaneously ventilated at exactly the same rate. So by constructing a switching mechanism to divert the excurrent flows from different chambers in various directions it is possible to get the analyzer to sequentially measure a series of chambers. A typical configuration might include eight chambers, but ones with 16 and even 32 chambers are also available. The key point about these systems is that there is still only 1 analyzer and that analyzer cannot measure two chambers at the same time. So each mouse in the system is measured for only part of the time. Theoretically one might imagine in an eight chamber system each mouse is measured only 1/8th (12.5%) of the time, but in fact this is not the case because in a switching system it is necessary to have a period between each switch where the system purges the gas currently in the system from the previous animal. So for example if it takes 2 min to purge the system and the chamber flips between chambers every 3 min then the system will be purging for two-thirds of the time and measuring for only one-third. Each animal will then be measured for 1 min every cycle around the eight chambers which will take 8 min × 3 min to complete. Hence instead of being continuously monitored as in a single chamber-single analyzer system each animal is measured for just 1 min every 24 min (4.1% of the time). Clearly as the numbers of chambers increases this "measurement" becomes less and less representative. For a 32 chamber system on the same 3 min cycle each animal would be measured for 1 min every 96 min (1.04% of the time). If the time/chamber is increased before flipping to the next one in the sequence then the percent time spent purging is reduced. For example if the time/chamber was increased from 3 to 10 min then the system would be purging only 20% of the time. Hence the animal would be measured for 8 min. However, that 8 min would come around much less frequently. In an eight chamber system only once every 80 min. The animal is now measured 10% of the time instead of the 4.1% of the time on the 3 min cycle, but the measurement depends on how representative that continuous 8 min is of the whole 80 min. Most researchers have tended to go for more rapid sampling to get a more even spread of the measured minutes across the whole measurement time. Hence while it may appear that these multichannel systems are measuring 8, 16, or 32 animals, in reality they are often measuring nothing, because they are purging the system, and when they do measure something they

still only measure one animal. It has been argued that these factors compromise the use of such systems for accurate determination of energy expenditure (Even and Nadkarni, 2012).

These two types of system are actually designed to do very different jobs. The single channel one chamber one analyzer system, generally using a very small chamber is ideally designed for making measurements of components of the energy balance such as Basal metabolic rate (BMR), or thermoregulatory costs, or the costs of physical activity. They are also ideally suited to measuring the acute impacts of treatments with drugs or with compounds believed to impact on energy metabolism (e.g., Hoggard et al., 2004; Valle et al., 2008). However these systems are unable to measure daily energy demands because the chamber is too small for the animal to live in for any protracted period. The multichannel systems where several chambers feed into a single analyzer are designed to make exactly this latter type of measurement. In this case the chamber volume is much larger so that it can contain a food hopper and water dispenser, space for a nest and also space to allow the animal to move around. The larger chamber with a slow washout and infrequent monitoring is poorly suited to measuring the detailed components of energy metabolism. However this system is ideal for measuring daily energy demands. The slow washout characteristics integrate the animals metabolism over time, this is compatible with the infrequent chamber monitoring. In recent years there have been attempts to decompose the measures from these multiple chamber systems into the components of metabolism (van Klinken et al., 2012). At present these methods are poorly advanced and the resultant accuracy cannot match frequently sampling single chamber-single analyzer systems (Even and Nadkarni, 2012). However, multichannel systems have recently entered the market that work on a one chamber one analyzer principal (the Promethion system from Sable systems is an example). In these systems each chamber IS monitored continuously, generally also with a continuous reference measurement. Such systems are superior to the standard switching systems based on a single analyzer monitoring multiple chambers and using this system it may be possible to get the best of both worlds – a good daily energy expenditure measurement with an accurate decomposition of the components.

CORRECTING MEASUREMENTS TO STANDARD TEMPERATURE AND PRESSURE DRY AND SI UNITS

Gas volumes change with temperature and pressure. Hence when we calculate the oxygen consumption of an animal by measuring the flow volume (as opposed to mass) and multiply that by the concentration differences in the airflow, the result that we get is dependent on the ambient temperature at which the air flow rate is controlled and the barometric pressure at the time the measurement is made. This is not the case if the mass of the flow is determined. Since temperature and pressure may vary over the time course of a measurement it is also often necessary to measure these at the start and end of each measurement and to assume linear drifts in these parameters as well. Alternatively some machines have an ambient temperature and pressure compensation system fitted. This basically measures the ambient pressure and temperature continuously and then exerts a back pressure into the flow to simulate a constant pressure of 760 mmHg and a temperature of 0°C. If such a device is not fitted the correction to standard temperature and pressure for dry air must be performed. In both cases the resultant oxygen consumption should be referred to as VO_2 STPD. The SI unit for volume is the liter. For mice respiratory gas consumption or production is normally expressed in units of ml/min, or L/h. If corrections for temperature and pressure are made automatically by the instrument then it is important for the user to ascertain that the Standard Temperature and Pressure values that are used by the software of the indirect calorimetry system to derive the flow are equal to the STP values used in the tables in literature that contain the coefficients of energy expenditure/volume of O_2 or CO_2. In more recent years to avoid any confusion about standard temperatures and pressures it has become common in the comparative physiology literature to express oxygen consumption or CO_2 production in mols of oxygen or CO_2/unit time.

The SI unit for energy is the joule. Although the use of calories is common this is not an SI unit. The SI unit for the rate of energy expenditure is the Watt. One Watt is equal to 1 J/s. Energy expenditures of mice measured over periods of minutes and hours should normally be quoted in Watts. However, because the time base of the Watt is the second this gives a poor idea of the level of expenditure over a whole day, which in many cases is the variable of interest. Hence daily energy expenditures should normally be quoted in kJ/day.

PRACTICAL ISSUES

BMR, RMR, AND RMRt

Basal metabolic rate, occasionally called BEE (basal energy expenditure), was introduced early last century to standardize measurements of metabolism across different species. The basic requirement for a measure to qualify as basal is that the organism should be at rest, alert (i.e., should normally not be sleeping), post-absorptive (i.e., not digesting food), not growing or reproducing, at a temperature within the thermoneutral zone and measured during the quiescent phase of its diurnal cycle. Although not initially prescribed it is also generally assumed that this animal it at its normal body temperature (euthermic) and in the quiescent phase of its daily cycle (Aschoff and Phol, 1970). Effects of time of day on the metabolism of mice have been known since at least the 1940s (Fuhrman et al., 1946). To qualify as a measure of resting metabolic rate the only criterion is that the animal should be at rest and euthermic. Many measurements of metabolic rate in mice fall between these two limits. That is they meet the criterion of being at thermoneutral, but it is not entirely clear if the animals are post-absorptive or not. Speakman et al. (2004) suggested the term RMRt should be used for these measurements, which is additionally useful for measurements that are otherwise basal, but made on growing or reproducing animals.

A key issue in measuring BMR in mice is the time needed to starve a mouse to make sure it is post-absorptive. Initially it was assumed that for most animals it was necessary to starve them overnight (Kleiber, 1961). However, not feeding overnight is a major energetic challenge to most small mammals the size of a mouse and in response they enable many defense mechanisms to conserve energy. This includes suppressing metabolic rate and lowering the body temperature. This leads to a situation where the requirements for BMR start to become mutually incompatible.

The longer an animal is starved the more likely it is to be post-absorptive, but the less likely it is to be euthermic (see also Gallivan, 1992; Speakman et al., 1993; McNab, 1997 for discussion of this trade-off in the measurement of cetacean and soricid metabolic rates). In consideration of these issues many recent measurements for mice and other small rodents have used much shorter periods of starvation prior to the measurement of BMR in the range of 4–5 h (Ksiazek et al., 2004; Sadowska et al., 2009; Zhao et al., in review).

Although chamber size does not enter into the calculation of metabolic rate it has a large impact on metabolism measures. This is because the chamber acts as a mixing box for the respiratory gases. The concentration of oxygen and CO_2 exiting the chamber is therefore a reflection of the integrated pattern of the oxygen consumption and CO_2 production of the animal over a period of time. The duration of this time depends on the chamber size, chamber design, and the flow rate. The lower this time is the more closely the excurrent gas flow reflects the instantaneous metabolism of the animal being measured. In theory if a pulse of CO_2 was introduced into a chamber at time 0, and there was perfect mixing in the chamber then the concentration of CO_2 in the excurrent flow would decline exponentially back toward the baseline. The half life of this exponential decline is a measure of the chamber washout characteristics. It is dependent on the chamber volume and the flow rate. Higher flow rates and smaller chambers lead to faster washout characteristics. By making the washout faster the measured oxygen consumption more closely reflects the instantaneous metabolic rate of the animal being measured. There are however several trade-offs to be considered. If the chamber is too small it may be restrictive and the animal may be stressed by the confinement and have an elevated metabolic rate (Pertwee and Tavendale, 1977). So chamber volume can only be reduced to a certain extent. The smallest chambers we have successfully used for measuring BMR in mice are cylindrical chambers with a diameter of 6 cm and a length of 10 cm giving a chamber volume of 283 ml. The washout time can also be reduced by increasing the flow rate. However as the flow rate is increased the difference in oxygen and CO_2 contents between incurrent and excurrent gas streams gets smaller and the consequence is potentially reduced precision in the estimated difference. For example, if one was measuring a mouse with a resting metabolic rate of 0.6 ml/min using a flow rate of 1,000 ml/min, the difference in oxygen concentration between the incurrent and excurrent flows would be only 0.06%. Ideally the difference between incurrent and excurrent oxygen and CO_2 concentrations should be maintained above 0.2%, and ideally in the range 0.2–0.8%. This sets a limit on improving washout by increasing flow rates. However chamber volume and chamber flow rate are not the only factors influencing washout rates.

By strategically locating the inflow and outflow of the chamber, mixing in the chamber can be maximized. However, chamber design may have a significant impact on washout characteristics. Square or oblong chambers may have dead spaces in the corners that retard mixing in addition adding any form of complexity inside the chamber may also create dead spaces that impede mixing of the gases inside the chamber. Ideally for BMR and RMR measurements it is best to have as simple a chamber as possible. We use cylindrical chambers measuring 8 cm in diameter and 25 cm long with a perforated floor in the bottom that keeps the mouse separated from any feces or urine it may produce. The volume is 1,257 ml. Using a flow rate of 300 ml/min the half life for the washout is about 2.5 min, and the difference in oxygen concentration between inflow and outflow for a mouse consuming 0.6 ml/min is about 0.5%. This gives a balance between minimizing washout time, not restricting the animal too much, and maintaining a large enough incurrent-excurrent concentration difference of the respiratory gases to get a precise estimate.

The duration of a BMR measurement needs to be long enough for the animal to completely settle down within the chamber. It is often suggested that animals should be familiarized with the chamber environment on a number of trial runs prior to the actual measurements but we have not found any evidence that there are systematic differences in BMR measures between the first, second, third, and fourth experiences in the chamber (Duarte et al., 2010) using mice that had no prior exposure to the environment. Hence this preconditioning does not seem necessary. Four typical patterns of metabolic rate and simultaneous physical activity and body temperature during a respirometry measurement in a chamber like that described above are shown in **Figure 1** (from Duarte et al., 2010). These measurements show some common features of all measurements we have made on mice. Initially there is much physical activity in the chamber. This seems to be exploratory and does not decline with repeated measurements. During this period the body temperature is also elevated to between 38 and 38.5°C. This phase generally lasts for about 30 min to an hour. The mouse then settles down (normally curled up and stationary) and the metabolic rate gradually declines. The decline in metabolism reflects a slow decline in body temperature to a stable level between 35.8 and 36.5°C. Both metabolic rate and body temperature normally reach a stable minimum after about 2 h. The animal may wake up move around and then go back to sleep. These periods of elevated physical activity correspond to periods of increased metabolic rate and body temperature. These are particularly noticeable in the traces for animals 14, 22, and 42. Occasionally there are dips in the metabolic rate (see especially the trace for animal 45). Not all animals show these dips and they are not observed on all repeat measurements in the same individual.

Measurements of BMR are generally made as the lowest observed metabolic rate over a pre-defined measurement period (for example 5 min – see Discussion below over the choice of this interval). However, the experimenter has a choice how long to leave the animal in the chamber waiting for a low 5 min period to be observed. In theory the longer an animal is left the lower the measurement will be. This is because if actual metabolic rates follow a Gaussian distribution around some mean value and one draws samples at random from this distribution, then the more samples you take the more likely you are to get a lower one than the previous lowest you observed (Hayes et al., 1992). We have looked at a large number of metabolism measurements in both mice and bank voles and found that the lowest 5 min measurement declines as the measurement duration is increased, mostly because of the change in physical activity, but this decline is not significant after about 2.5 h in mice. We therefore make our BMR measurements over 3 h. In bank voles we use 4 h because the decline in the estimate remains significant until about 3.5 h. Longer measurements

may in any case become an issue as the animals are deprived of water whilst in the chamber.

The rate at which samples are collected from the analyzer and averaged is also a variable under experimenter control. Modern Analog to Digital conversion cards make thousands of conversions every second, so even sampling at a rate of several measurements/second is feasible. However, if the chamber washout characteristics mean that the chamber half life is measured in minutes, these high frequency measurements are not independent of each other, and variation between them more likely reflect equipment noise than any biological phenomenon. With a washout time of about 2.5 min in our system we typically make time-averaged measurements over 10–30 s intervals. In switching systems where a single analyzer pays attention to several chambers sequentially making a large number of high frequency measurements over the short interval that the chamber is being measured does not compensate for the short time each chamber is actually measured, as these high frequency measures are pseudo replicates.

As mentioned above, most researchers characterize BMR as the lowest measured metabolic rate over some pre-defined period (e.g., 5 min). The estimate obtained however is theoretically (Hayes et al., 1992) and practically dependent on the duration of this interval. **Figure 2** shows the empirical relationship between the estimated minimal metabolism and the duration over which the measurement is averaged for a typical sample of 50 metabolic rate measurements in mice (data from Vaanholt et al., 2012). There is a positive curvilinear relationship for all the measurements, but this seldom reaches an asymptote. This curve probably reflects the fact that at short intervals one is picking up stochastic variation in metabolism and/or noise in the equipment/system. These stochastic variations get smoothed out by extending the duration, but become more likely to then include brief periods of activity or small movements. Because there is no clear asymptote in this relationship the choice of the duration over which to average is arbitrary. In our system, with a chamber washout of 2.5 min, a sampling time of 5 min seems a reasonable compromise between avoiding stochastic variation on one side and including minor activities on the other. This choice however is specific to our system. In systems with larger chambers and longer washouts there will be an illusion of greater robustness to the choice of sample duration simply because these stochastic variations and minor activities are integrated by the chamber and not therefore detectable by the analyzer.

One feature evident from **Figure 1** is the transient reductions in metabolic rate that are occasionally observed (see especially for animal 45). The cause of these reductions are unclear but they do not appear to be a machine artifact as they are never observed if the chambers are operated without an animal present and they consequently seem to be a real feature of metabolism. One possibility is that the animals simply stop breathing, and become apneic for a short period. Apnea is frequently observed in animals during torpor when the metabolic rate is extremely low (e.g., Hays et al., 1990; Thomas et al., 1990) and these data suggest it may also transiently occur during euthermy. Whatever the cause these dips in the record mean that any estimate of metabolism using an algorithm to detect the lowest × minutes of metabolism will always home in on the region surrounding such a phenomenon. This may consequently be a completely unrepresentative measure of the BMR. To avoid this problem we have started to also use an algorithm that detects the least variable *n* minutes of metabolism. This finds the most stable period of measurement, which is generally a period of low metabolism without any dip in it. If there is a discrepancy between the absolute lowest and the least variable we choose the least variable.

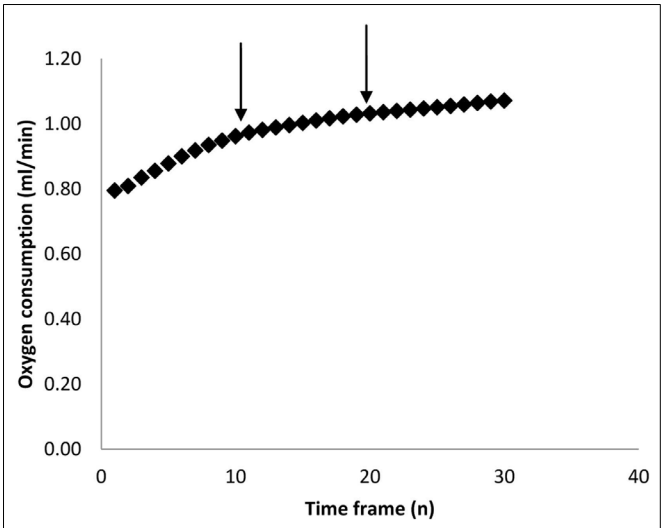


FIGURE 2 | Calculated minimum oxygen consumption over increasing time frames (*n* measurements). Each measurement lasted 30 s. Data are averaged across 43 individual MF1 mice involved in a study of weight loss on calorie restricted diets (Vaanholt et al., 2012). The arrows mark 5 and 10 min intervals which we have used in previous publications.

A key requirement for the measurement of BMR (or RMRt) is that the animal is at a thermoneutral temperature; that is it is measured within the thermoneutral zone (TNZ). Because evaporative water loss increases as one moves from the lower margin of the TNZ to the upper margin the most desirable temperature at which to measure BMR is around the lower critical temperature. This is particularly because in most metabolic chambers designed for BMR measurements the animals do not have access to water. In mice the T_{lc} has been estimated for various strains and is generally between 26 and 30°C (Hussein, 1991; Gordon, 1993; Speakman and Rossi, 1999; Selman et al., 2001; Golozoubova et al., 2004; Meyer et al., 2004; reviewed in Speakman and Keijer, 2013).

Several studies have previously addressed the repeatability of BMR in small mammals including mice (Labocha et al., 2004; Russell and Chappell, 2007; Boratynski and Koteja, 2009; Duarte et al., 2010) and other energetic measurements such as DEE (Speakman et al., 1994; Fletcher et al., 2013). Repeatability of the measurement of BMR (or RMRt) in mice is important for two reasons. First repeatability sets a limit on heritability. Second, by knowing the repeatability of a measurement we can evaluate using power analysis the required sample size to detect a real difference in BMR following a given treatment in a repeated measures design (see below under analytical considerations). We have previously measured the repeatability of RMRt in the mouse and found that the

measure is highly repeatable when measurements are separated by periods of about 15 days (Duarte et al., 2010). However, in part this repeatability is because there is a positive relationship between body mass and metabolic rate (**Figure 3A**) and animals tend to be consistent in their body masses. Nevertheless, if the residual metabolic rates are calculated (deviations from the fitted regression line between metabolism and body weight) these also show high repeatability when the interval between measurements is relatively short (**Figure 3B**). Over longer periods the repeatability is dependent on what the animal does between the measurements. In particular if the animal is female and goes through a cycle of reproduction then the repeatability is considerably reduced. However, in female animals that do not reproduce it remains high even if the interval between measurements is >100 days (Duarte et al., 2010).

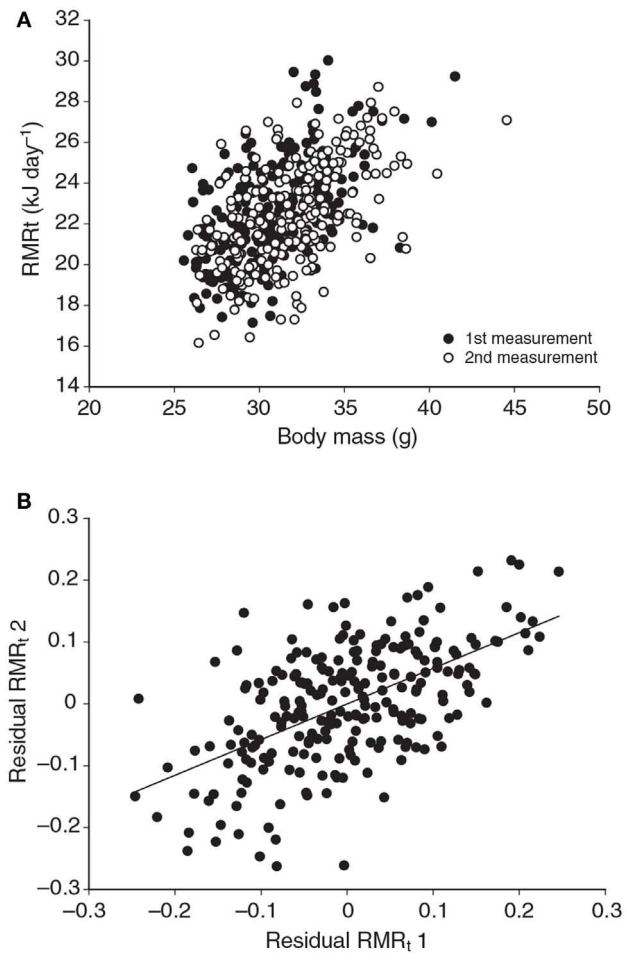


FIGURE 3 | (A) Relationship between RMRt (resting metabolic rate measured at thermoneutral temperatures) and body mass for 323 mice measured on two occasions separated by about 15 days. There was a strong effect of body mass at both time points. **(B)** Residual measurements of RMRt, with the effect of body mass removed, measured at time 1 plotted against the same data at measurement time 2, for the same 323 mice. Residual RMRt was highly repeatable over this short timescale (data from Duarte et al., 2010).

THERMOREGULATION

Issac Newton was among the first scientists to observed the temperatures of different sized bodies as they were cooled and warmed and kept in different ambient temperatures. The standard Newtonian model for the thermoregulatory response curve of an endotherm is shown in **Figure 4**. The expectation is that metabolism will increase at temperatures below T_{lc} in an almost linear fashion until the animal reaches a maximal metabolic rate. The gradient of this relationship between resting metabolism and ambient temperature is the whole body thermal conductance, and it extrapolates to the body temperature on the x-axis. Lower ambient temperatures than the temperature at which metabolism reaches a maximum lead to reduced metabolism because the maximum is unable to sustain body temperature and hence the reduced body temperature feeds back to reduce the metabolism until some dynamic equilibrium is reached. Above T_{lc} the BMR provides too much heat to balance thermoregulation requirements and hence evaporative water loss increases to dissipate this excess. At some point (the upper critical temperature) the animal must effect other mechanisms that paradoxically increase metabolism and lead to exponential increases in evaporative water losses and elevated body temperatures.

I could find no complete curves for mice in the literature although many incomplete curves have been published: **Figure 5** shows the thermoregulation response curve for male MF1 mice, not acclimated to cold conditions. For MF1 mice this curve indicates a T_{lc} of about 26°C, and a maximal cold induced metabolic rate about 5.6× the basal metabolism attained at a temperature of −5°C. Based on this evidence mice seem to conform closely to the Newtonian cooling model. As noted above previous estimates of the lower critical temperature range between 26 and 30°C. These temperatures correspond closely to the temperatures that mice prefer (26–29°C) when given a choice (Gordon et al., 1998; Gaskill et al., 2009, 2012).

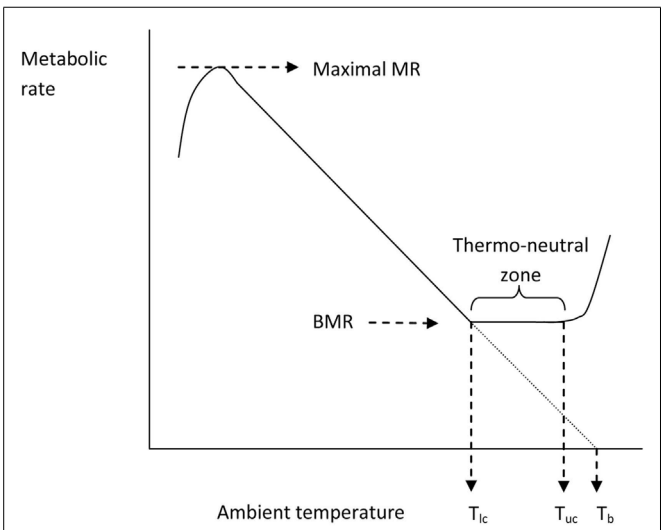


FIGURE 4 | The classical Newtonian cooling model describing the effect of ambient temperature on the metabolic rate of an endotherm like the mouse (after Scholander et al., 1950). T_{lc} = lower critical and T_{uc} = upper critical temperatures that bound the thermoneutral zone.

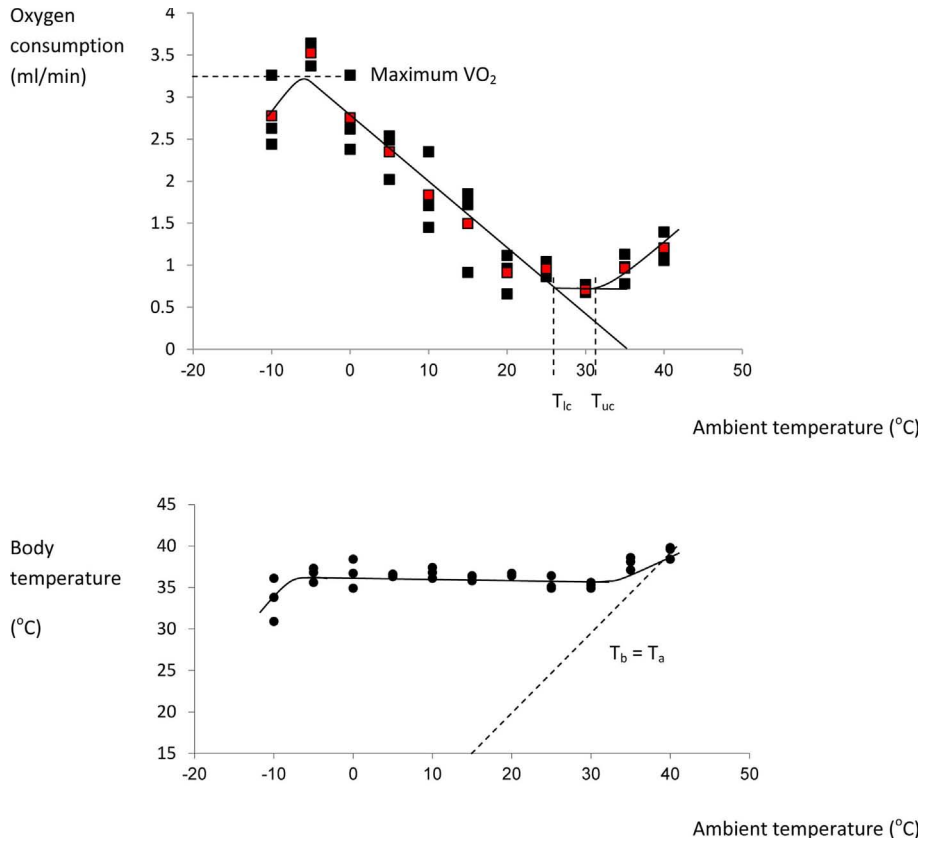


FIGURE 5 | Thermoregulation curve for the male MF1 mouse. Individuals were measured for 3 h at 30 s intervals at each temperature in a single chamber-single analyzer system and the reported oxygen consumption was the lowest continuous 5 min period over the 3 h. Each point represents a different individual. Red points are means at each temperature. Body temperature (lower plot) was measured after the individuals exited the chamber. The characteristic temperatures bordering the thermoneutral zone (lower critical: T_{lc} and upper critical: T_{uc}) are indicated on the upper plot, and the line of equivalence where body temperature (T_b) equals ambient temperature (T_a) is shown on the lower plot. (Data from Speakman, J. R., unpublished).

There are two basic mechanisms by which mice generate the heat to sustain their body temperatures below thermoneutrality. They use the heat generated by muscular contraction, i.e., they become physically active or they shiver, or they generate heat by non-shivering thermogenesis. Non-shivering thermogenesis is generally presumed to originate primarily in brown adipose tissue as a result of the action of uncoupling protein 1. The balance between different sources of heat is strongly affected by the animals previous history of cold exposure. In a naive animal exposed to the cold the response is almost completely from shivering. However, in animals that have been exposed previously to the cold for protracted periods the response is almost entirely due to non-shivering thermogenesis.

The curves in **Figure 5** highlight that in normal laboratory conditions where mice are maintained at 19–21°C they are held under perpetual mild cold stress (4–6°C below thermoneutral). It has been suggested that keeping mice under these conditions may be a poor reflection of the situation in humans who live almost perpetually at thermoneutral temperatures (Swoap et al., 2008; Cannon and Nedergaard, 2009, 2011; Lodhi and Semenkovich, 2009; Overton, 2010; Karp, 2012). Housing temperature does seem to have an effect on some metabolic responses and genotype effects (see also Pincede et al., 2012 for the effects of ambient temperature on nociceptive tests in mice). For example in the UCP-1 KO mouse, studies at 21°C exposing them to a high fat diet did not reveal any phenotype relative to wildtype mice (Enerbäck et al., 1997) but these effects were potentially confounded by the background of the strains used. A later study revealed that when on a C57BL/6 background knocking out UCP-1 actually led to a paradoxical protection from diet induced obesity, which was absent at 26°C (Liu et al., 2003). Moreover, when these mice were maintained at 30°C they became obese relative to wildtypes, even when feeding on chow, an effect that was amplified when fed on a high fat diet (Feldmann et al., 2009). Although this demonstrates a strong effect of housing temperature, the reasons for the effect remain uncertain and opposite to that expected if UCP-1 is the main effector of non-shivering thermogenesis. Hence one might imagine its impact of its absence on weight gain would be greatest at the lower temperature since UCP-1 mediated metabolism contributes virtually nothing to BMR in mice at thermoneutrality

(Golozoubova et al., 2001, 2006). Potentially other sources of non-shivering thermogenesis may be involved in these confusing responses of the $UCP^{-/-}$ mice including for example from muscle mediated via sarcolipin (Bal et al., 2012). Given the responses to loss of UCP-1 span the whole range from protection from diet induced obesity at 21°C to susceptibility at 30°C, the question remains which of these responses most closely reflects the situation in humans. Speakman and Keijer (2013) compared the thermal response curves of mice and humans and concluded that for single housed mice the optimal temperature for comparison to humans would be around 23–25°C. At this temperature loss of UCP-1 seemed to have no impact on mouse susceptibility to a high fat diet induced obesity (Liu et al., 2003).

The whole body thermoregulation curve (**Figure 5**) cannot be used to generate an indication of brown adipose tissue or non-shivering thermogenesis as the heat to maintain body temperature is generated from multiple sources by multiple mechanisms. For the same reason it is also the case that acutely exposing an animal to the cold (e.g., 4°C) also cannot tell us anything much about its capacity for non-shivering thermogenesis (see also Cannon and Nedergaard, 2011). To measure non-shivering thermogenesis the procedure is generally to keep the mouse at a fixed ambient temperature (normally 30°C to prevent any shivering) and inject the mouse with noradrenaline to activate non-shivering thermogenesis via beta adrenergic receptors in the brown adipose tissue. Since beta adrenergic receptors are more widely distributed in the body and the dose required to stimulate the BAT is also sufficient to stimulate these other receptors there is some stimulation of non-shivering heat production in other tissues than BAT. Cannon and Nedergaard (2011) suggest this is purely a pharmacological effect that has no adaptive significance in the live animal. The extent of this non-BAT stimulation of metabolism by NA can be evaluated by comparing genetically manipulated mice with no UCP-1 to wild type mice with native UCP-1 (**Figure 6**). This suggests that for mice with no history of cold exposure almost all the heat produced following NA injection is from non-BAT sources, but for mice that had experience of cold previously, the contribution is much less at around 10–20%.

The procedure for the NA test of non-shivering thermogenesis is described in Cannon and Nedergaard (2011) and briefly summarized as follows. Animals can be measured awake (e.g., Jansky, 1973; Jackson et al., 2001a,b) but commonly they are first treated with a barbiturate based anesthetic. If a conscious animal is used the animal is placed into a respirometry chamber to obtain a baseline basal measurement (normally about 3 h: see above). Anesthetized animals show no physical activity and can be measured over a much shorter pre-injection period. The argument for anesthetizing the animals is that metabolic rate may be elevated as a stress response to injection in conscious animals. After the baseline measurement is complete the animals are then removed from the chamber and injected with NA by the dorsal subcutaneous route so that the injectate floods over the interscapular brown adipose tissue. IP injections generate a much poorer response. A dose response curve was produced by Heldmaier (1971) which suggested doses over 1.0 mg/kg elicit a maximal response. Doses higher than 1.5 mg/kg can be fatal (pers. obs.). The animal is then

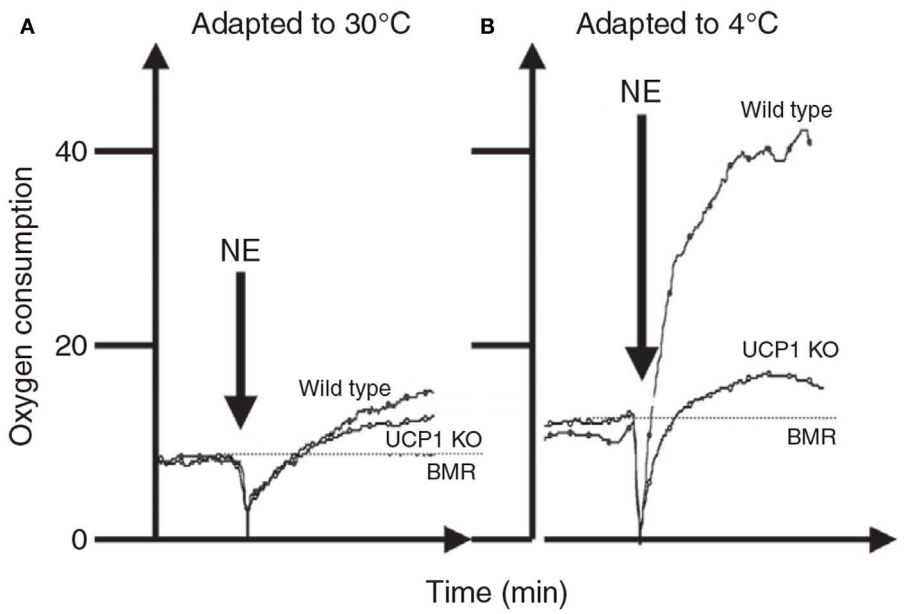


FIGURE 6 | The response of mice to norepinephrine injections. In **(A)** the mice were maintained prior to the experiments at 30°C. There was only a very small response to the NE and it did not differ much between mice that have and do not have UCP-1. In **(B)** the mice were kept prior to the measurements at 4°C. Here the response to NE in the wild type mouse was much greater but that of the UCP-1 KO mouse similar to that in mice housed at 30°C. This suggests that all of the thermoregulatory conditioning to increase non-shivering thermogenesis by housing animals in cold conditions is mediated via UCP-1. Units for oxygen consumption were not stated and no time details were provided on the *x*-axis in the original source (data from Cannon and Nedergaard, 2011).

immediately returned to the respirometry chamber. Normally, awake animals remain completely quiescent after the injection. This is probably because any physical activity would exacerbate the induced heat production and make them at risk of fatal hyperthermia. Since stressed animals would normally manifest their stress by elevated physical activity the presumed impact of stress in the measurement of NA-induced metabolism in conscious animals has probably been overemphasized.

Following return to the chamber there is a large increase in metabolic rate which reaches a peak and then subsides – a typical example is shown in **Figure 7A** (Jackson et al., 2001a; Arch et al., 2006). The shape of this curve depends completely on the chamber characteristics in which the measurement is made. In a fast washout system the peak reached will be much higher than in a slow washout system. Comparing peak responses across studies is therefore complicated by lab specific details of the respirometry systems utilized. There are methods to get over this problem discussed in more detail below in the context of measuring physical activity costs. The results of applying such a conversion to the data in **Figure 7A** is shown in **Figure 7B**. These data show that even when a fast washout small volume chamber is used the "instantaneous" estimates of metabolism can still be substantially higher than the actual measurements if the metabolism is changing rapidly. In this case the difference was 40%. The area under the curve is a chamber independent measure of the response that does provide a possibility for comparisons but is generally never reported as measurements are frequently discontinued before the metabolism returns to baseline. The instantaneous peak response to NA injection is strongly dependent on body mass (**Figure 8**; Jackson et al., 2001b) which means the body mass effect must be taken into account when comparisons are made between different genotypes (see below under analytical considerations). Attempts to quantify the NST activity in response to NA using infrared thermography to quantify the surface temperature rise above the iBAT have been attempted (Jackson et al., 2001b) with limited success.

PHYSICAL ACTIVITY

Mice have a range of physical activities but their primary mode of locomotion is running. The cost of running in mice can be measured using a tread-wheel apparatus within the respirometry chamber. This allows the speed of running to be manipulated by the experimenter and the consequent costs of locomotion at each speed derived. The main requirement in such procedures is that the animal reaches a steady state performance of the behavior for a long period relative to the washout characteristics of the chamber. That is the animal needs to run continuously for several minutes so that a stable running metabolic rate can be measured. Mice in captivity (and probably also in the wild) seldom run for such protracted periods so their behavior may not always be adequate, and some training in the apparatus is normally necessary before the animals will perform the required behavior. We have found that varying the speed during training seems to improve the behavior, perhaps because mice normally oscillate the speed at which they move.

The relationship between running speed and metabolic rate in mice is linear (e.g., Figure 9: Schefer and Talan, 1996). Extrapolating the relationship back to the *y*-axis at a running speed of zero generally yields a value that exceeds the measured rate of basal metabolism. In the example shown in **Figure 9** the extrapolated *y*-axis intercept was between 5,000 and 6,000 ml/kg/h but the actual measured resting metabolic rate was between 2,700 and 3,200 ml/kg/h. This difference has been often interpreted as a "postural" cost of locomotion. The data in **Figure 9** also illustrate that the cost of locomotion depends on subject age and that the RER is also dependent on running speed with higher speeds being associated with elevated RER values. In this case the division of the values by body mass could mean the age effect was an artifact of a body mass difference (see below under analytical considerations), but in fact the aged mice were lighter than the adult mice so this age effect was not an artifact.

More often, however, rather than the costs of locomotion, researchers are interested in how much of the daily energy budget of a mouse can be ascribed to "general" physical activities. This might include "locomoting" but would also include many other behaviors such as grooming, climbing on the cage bars, and eating, etc. Ascribing a cost to this sporadic data from chamber studies is difficult for two reasons. First, the behaviors are highly variable and most likely have different costs. Second, if a mouse

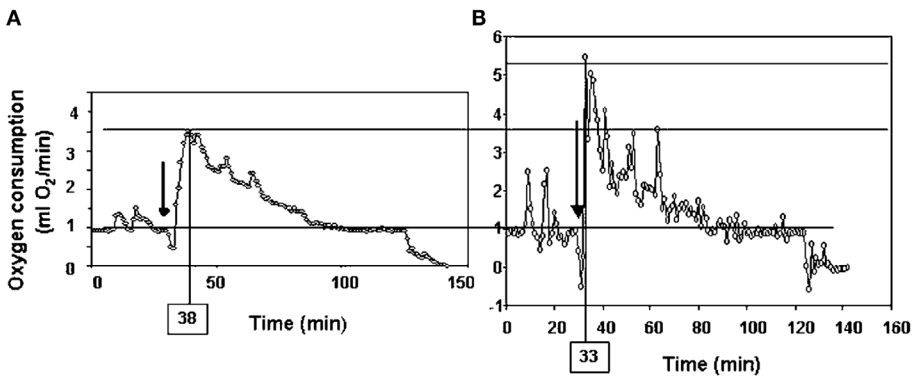


FIGURE 7 | Time course of the response of a small rodent (the short-tailed field vole: *Microtus agrestis*) to injection of Norepinephrine. The plot in **(A)** is the raw data from the respirometry chamber. In **(B)** the data have been mathematically manipulated to reconstruct the instantaneous changes in metabolism (figure from Arch et al., 2006 and original data from study by Jackson et al., 2001a).

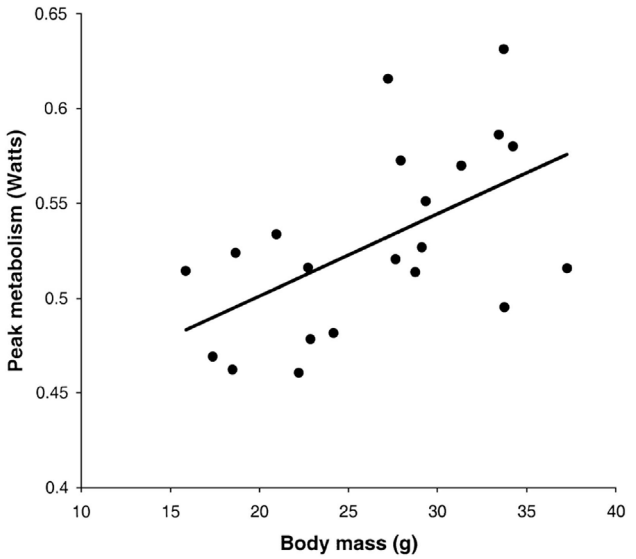


FIGURE 8 | Instantaneous peak metabolic rate following norepinephrine injection in relation to body mass. Data are for a small rodent about the same size as a mouse (the short-tailed field vole: *Microtus agrestis*; Jackson et al., 2001b).

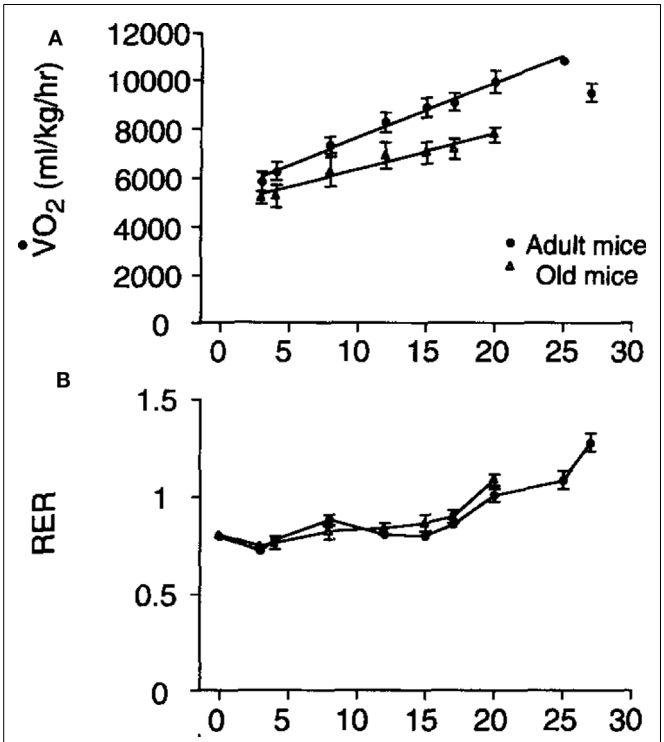


FIGURE 9 | (A) Mass specific oxygen consumption in relation to running speed for adult and aged mice and **(B)** the patterns of respiratory exchange ratio (RER) in the same animals (from Schefer and Talan, 1996).

performs a behavior within a metabolic chamber the record of its metabolic rate by the analyzer is not an instantaneous reflection of the actual metabolic rate. There is the mixing in the chamber to be considered plus the delay between the excurrent gas exiting the chamber and arriving at the analyzer. Thus the peak metabolic rate measured following a behavioral event is a poor reflection of the actual costs of the activity. Probably the first study to consider these issues was that by Bartholomew et al. (1981) who studied the warm-up metabolism in moths. They realized that the observed metabolic measurements could be used to reconstruct the actual time course of metabolism if information on the washout characteristics of the chamber being used were known, and the change in metabolism between measurements was used in addition to the actual measurements. This would enable reconstruction of the "instantaneous" estimates of metabolism. The procedure is known as deconvolution, and full details of it can be found in Arch et al. (2006) and Lighton (2008). The effects of applying this approach on the metabolism curve following injection by NE in **Figure 7A** are shown in **Figure 7B**. These data show that the "actual" peak metabolic rate was 40% higher than the highest measurement in the chamber and occurred much earlier.

An example of using this methodology was the study by Speakman et al. (1989) to measure the energy costs of echolocating behavior in small bats. By converting the actual measurements to the equivalent "instantaneous" estimates of metabolism it was possible to regress the echolocation behavior of the bats (pulses/minute) on the metabolic rate to work out the cost of echolocating. This still did not take into account of the lag between the excurrent flow leaving the chamber and being measured at the analyzer, so to account for this the regressions were performed stepping the metabolic rate measurements relative to the behavior measurements. This showed the maximal r^2 for the regressions corresponded to a lag of about 2 min, approximately corresponding to the expected lag based on the flow rate and system configuration.

In this latter application the behavior was very simple to correlate against the instantaneously corrected metabolism because the behavior could be easily characterized in numbers (echolocation pulses). For mouse behavior this is more problematical but fortunately a solution to the problem of characterizing mouse behavior as activity has been produced and this involves monitoring the movements of the animals and then converting these movements into "counts." There are different proprietary solutions to this problem based on different technologies for monitoring the movements and the data they generate is not equivalent. However, if the behavior of an animal is monitored while it is in a respirometry chamber and it is converted into "counts," then it would be a relatively straightforward matter to regress these counts onto the derived estimate of "instantaneous" metabolism in the same way as performed previously to estimate the cost of echolocation. In fact this has not yet been done, but instead some studies have regressed the counts of activity onto the "simultaneous" uncorrected metabolic rate estimates (e.g., Bjursell et al., 2008). The reason for this is because the systems used to perform this work have been switching systems where each chamber is monitored relatively infrequently, the chambers are large and the washout is relatively slow. Hence the refinement of making "instantaneous" estimates of metabolism cannot be performed, and the lag of the

system is small (seconds) relative to the time between measurements of each chamber (minutes). The resultant regression is used to estimate the costs of activity (gradient of the regression) and the RMR (intercept; e.g., Nonogaki et al., 2003) from data spanning 24 h or longer periods. One potential issue with this approach is that it assumes the baseline RMR is constant, yet we know that RMR will vary depending on the time of day (active and quiescent phases) and also on the thermic effect of food.

To overcome this issue a much earlier study of rats by Even et al. (1991) used an approach called Kalman filtering to reconstruct the varying baseline RMR in a situation where the rats were moving around freely in the respirometry chamber. This method has subsequently also been applied to mice (Deveaux et al., 2009). Full details of the approach are in Even et al. (1991). A potential issue, however, is that Kalman filtering requires a more frequent sampling of the metabolism than is generally available from the use of multiple chambers linked up to switching devices. van Klinken et al. (2012) devised a penalized spline regression method to attempt to reconstruct the time varying RMR and showed that with a sampling frequency of 10 min this provided an estimated time dependent RMR that was 1.7× more accurate than using the Kalman filtering approach, and 2.7× better than linear regression. However, a 10 min sampling interval would be a fast turnaround time in a multi-chamber switching device, and the estimated RMR became systematically less accurate as sampling time increased above 10 min. The relative standard deviation in the estimated activity costs was similarly very sensitive to the sample time. At present reconstructing activity costs from these chambers results in estimates that have poor accuracy (Even and Nadkarni, 2012) – although the situation is constantly evolving.

BICARBONATE METHOD

An alternative approach to measuring the costs of physical activity in mice is to use an isotope based technique called the labeled bicarbonate method (Hambly and Voigt, 2011). In this method the rate of CO_2 production is measured by injecting animals with a bolus dose of ^{13}C labeled sodium bicarbonate. This comes to rapid equilibration (<5 min) with the body bicarbonate pool and is then eliminated from the body exponentially in relation to the rate of CO_2 production – hence providing an indirect measure of metabolism (Hambly and Voigt, 2011). Because the label appears in expired CO_2 it can be easily and non-invasively applied by measuring breath samples. This technique has been primarily used to measure the energy demands of unencumbered flight in both birds (Hambly et al., 2002, 2004a,b), and bats (Voigt and Holderied, 2012; Voigt and Lewanzik, 2012). However, an earlier study was performed in mice to validate the method against indirect calorimetry (Speakman and Thomson, 1997) and it seems to give a reasonable estimate of metabolic rate over periods of 30–90 min. This could in theory be used to measure the energy demands of activity by monitoring what animals do over the measurement period and then assessing costs across several individuals using multiple regression techniques.

DAILY ENERGY EXPENDITURE

Although multi-chamber switching devices are relatively poor for the determination of the components of metabolism, especially BMR (see above), these machines really come into their own when faced with the issue of measuring long term energy demands like the daily energy expenditure. This is because in this application the fine time resolution needed for an accurate estimate of BMR is unnecessary, and a chamber is required that mimics as closely as possible the home cage environment. This is impossible to achieve using the sorts of Spartan low volume chambers that are necessary to accurately determine BMR where there is often no capacity to also provide the animals with food and water. Multichannel systems using large chambers have become increasingly sophisticated with the measurement chamber also being instrumented with sensors to monitor ambient temperature, physical activity levels, food, and water intake and body mass of the subject. With automatic baseline measurements it is feasible to leave animals in these chambers for periods of several days to obtain repeated measures of the total daily energy expenditure. In this circumstance having a measurement every 20 min or so is adequate to evaluate the total daily energy demands, and the slow washout characteristics that are consequent of having a large chamber relative to the flow rate, and a complex chamber design that further reduces the washout time is actually an advantage because this makes the sampled time point more likely to reflect an average over the more protracted period of metabolism. Several excellent machines in this respect are available the main ones being the CLAMS system produced by Columbus instruments, the Phenomaster system produced by TSE systems, Ltd., and the Promethion system by Sable systems, Inc. These will all provide an accurate estimate of DEE. If you require to decompose the metabolic rate into resting and active components algorithms are currently in development by the manufacturers to achieve this (see van Klinken et al., 2012) but they are currently insufficient to achieve the sorts of accuracy that is possible using a single analyzer-single chamber system and a small volume chamber (Even and Nadkarni, 2012). The exception to this may be the Promethion device which also uses single chamber-single analyzer approach that can then be analyzed using the Kalman filtering method advocated by Even et al. (1991) or the penalized spline method by van Klinken et al. (2012). However things are currently moving rapidly in this field and in future accurate decomposition of the total daily energy demands into the main two components (rest and activity) may be feasible. At present, however, the best advice would be to use these devices to get good estimates of DEE, but use single chamber-single analyzer systems to obtain specific components such as BMR and RMR.

One issue when using such systems is how many days the animal should be left in the chamber to provide a useful measurement. It is common practice to discard the first day since this may be contaminated by exploratory behavior in the novel environment and then leave the animals in the system for 5–8 further days of measurement. Some preconditioning to the system may also minimize the novelty effect (Tschoep et al., 2012). However, if mice are placed into the chamber a couple of hours before recording begins there is no significant effect of day over 7 days of measurement (**Figure 10**), suggesting that rejecting the whole first day of measurements may be overly cautious. The overall coefficient of variation (overall SD/overall mean) across repeated measurement days is about 3% (calculated from data in Muller et al., 2013).

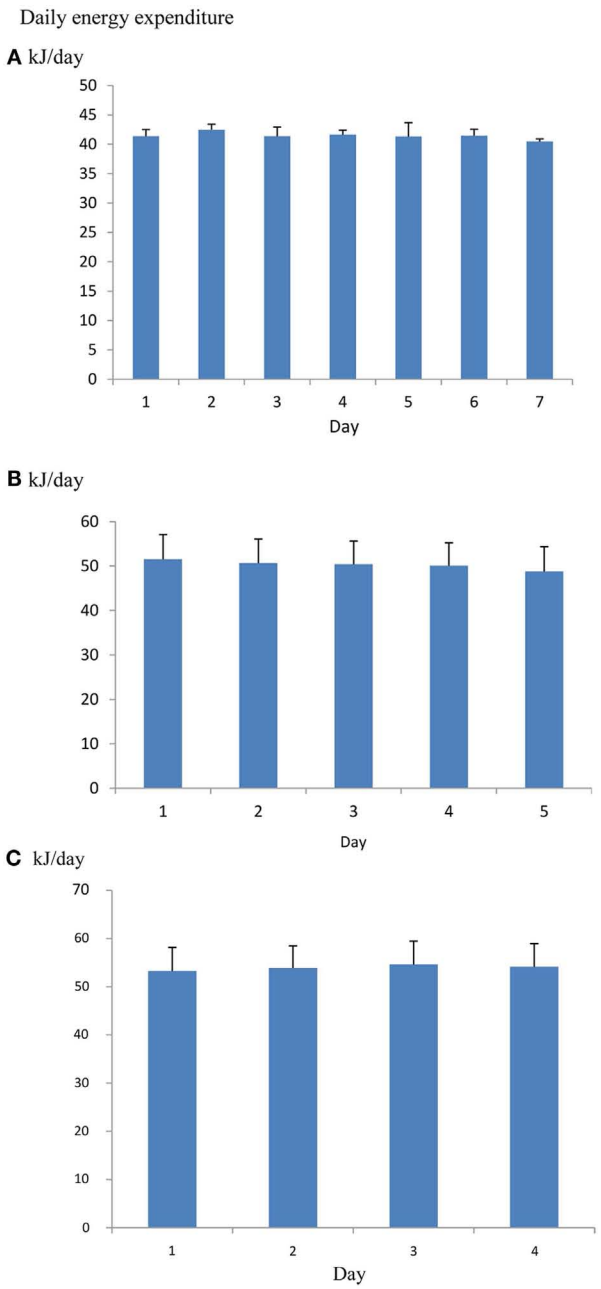


FIGURE 10 | (A) Mean daily metabolic rates (kJ/day) of four mice living in an Oxymax (CLAMS) open flow respirometry system continuously for 7 days. Error bars are standard deviations. Mice were placed into the system 2.5 h before recording started. There was no significant change in metabolic rate over the 7 days (Data from R. Sinclair and J. R. Speakman, unpublished). **(B)** the same type of data for 16 mice living in a TSE phenomaster system for 5 days (data from Timo Muller, pers. comm.) and **(C)** 15 mice living in a Promethion system for 4 days (data from Brent Wisse via John Lighton, pers. comm.). There was also no significant change over the 5 and 4 days respectively.

Hence averaging the metabolic rate across five consecutive days would yield an average estimate of DEE with a 95% confidence interval of also ±3% around the mean.

MEASURING DEE FOR ANIMALS IN SOCIAL SITUATIONS

In some situations measuring the daily energy expenditure of a mouse is impossible by the standard methods of indirect or direct calorimetry. These include for example the measurement of a female mouse when she is lactating. Measuring BMR of such a mouse can be performed (e.g., Johnson et al., 2001; Krol et al., 2003; Krol and Speakman, 2003a; Zhao et al., in review) by separating the mother from her pups and putting her into the chamber alone. This works for a BMR or RMR measurement, although there are some special considerations to made. Mice separated from their pups tend to be more active and take longer to settle down. In these circumstances a four rather than a 3 h standard measurement may be necessary. In addition lactating mice are often active and feed during the day. If they are food deprived for 4 h prior to the measurement followed by a 4 h measurement without food (e.g., Zhao et al., in review) this may potentially have an adverse impact on their lactation performance.

However, if a DEE measurement over 24 h is required then clearly separating the mother from her pups for this length of time would be impossible, yet the mother cannot be placed into the chamber with her pups because the resultant estimate is the summed energy expenditure of the combined mother and pups, not the mother alone. Other situations involve similar issues – for example measuring the energy demands of a single mouse when it is embedded in a social situation. For example, studies have been made of the consequences of social defeat on energy balance in mice (Bartolomucci et al., 2009). When a dominant and a subordinate mouse are housed together the dominant mouse appears resistant to weight gain but the subordinate mouse is not. These differences may be rooted in differences in their daily energy expenditure, but clearly separating the mice to measure them removes them from the paradigm that generates the difference we are trying to measure.

In these situations an alternative approach is needed. Two such approaches are the doubly labeled water technique and the heart rate technique (Butler et al., 2004). The heart rate method relies on the fact the fluctuations in energy demand are generally met by variations in heart rate. Hence it is possible to construct an individual calibration between energy metabolism and heart rate using standard indirect calorimetry with the animal in the chamber alone and then reconstruct the time course of energy demands over 24 h by logging the heart rate of the animal later when it is engaged in its social activities. This method has been used widely to measure the energy demands of free-living animals, but I am not aware of its application to date in the mouse. Technologically it is feasible because heart rate loggers capable of being implanted into mice are currently available (e.g., from DSL, Ltd., and from Minimitter).

The other technique, the doubly labeled water technique, is an isotope based method that relies on the differential elimination of isotopes of hydrogen and oxygen from the body (Speakman, 1997). Oxygen isotopes in the body water are eliminated by the dual flux of water and CO_2 through the body, while hydrogen isotopes are eliminated only by water. Hence the magnitude of the difference in the elimination of the isotopes is directly related to the CO_2 production, and particularly if RQ is known, the energy

metabolism. This method was actually developed in the 1950s in mice (Lifson et al., 1955). It has been subsequently refined, and the refinements validated in comparison to indirect calorimetry using voles (Speakman and Krol, 2005). This refined method has been applied in multiple studies in particular to measure the energy demands of lactating female mice (Johnson and Speakman, 2001; Johnson et al., 2001; Krol and Speakman, 2003b; Krol et al., 2007; Zhao et al., in review) and other small rodents (Wu et al., 2009; Simons et al., 2011).

Although these two methods come into their own when mice are in social systems and cannot be measured by indirect calorimetry, there is no reason why such methods could not be used in mice more generally to measure their energy demands over 24–72 h using DLW or much more protracted periods of days and weeks using the heart rate approach. Their complexity, for example requiring mice to undergo surgical procedures for the heart rate method, and the requirement for expensive mass spectrometry equipment for the DLW method, has probably inhibited their use to date.

ANALYSIS AND PRESENTATION ISSUES

DETECTING EFFECTS OF GENOTYPE

One of the commonest analytical situations in the study of mice is when one wishes to detect the impact of a genetical manipulation on the rate of energy expenditure. On the face of it this is a simple issue. One would measure a sample of mice representing each genotype and then compare their rates of energy utilization (Watts) using standard statistics such as the *t*-test or Analysis of variance (ANOVA). The problem is that generally when there has been an impact on the energy demands this is translated into a difference in body weight. Body weight is one of the key factors driving the rate of energy expenditure. Bigger animals have more metabolizing tissue and expend more energy. Hence if a difference is detected in the rate of energy expenditure this may be a secondary effect of the altered body weight, rather than a primary effect of the genotype alone.

A frequently used approach to try and rectify this effect is to simply divide the energy metabolism by the body weight to generate values of energy expenditure/gram (Watts/g). Butler and Kozak (2010) highlighted 10 very high profile papers in the top scientific journals where this method had been used, and Tschoep et al. (2012) reviewed over 50 articles on energy metabolism in mice and found that this approach had been used in almost 70% of them. This approach, however, only normalizes for the effect of body mass when the intercept of the relation between metabolic rate and body mass is at the origin. In the case of measurements of energy metabolism this is seldom the case. The reason why such relationships do not normally pass through the origin is because mice are made up of different tissues that metabolize energy at very different rates. In particular *in vitro* estimates of the energy metabolism of fat and skeletal muscle are substantially lower than for tissues like the liver, kidneys, heart, and brain (Krebs, 1950; Elia, 1992). When an animal grows larger it generally does not grow each of its tissues in direct proportion to each other (isometrically), when it loses weight it will generally draw more on adipose tissue than lean tissue, and differences between strains or genotypes also include changes in the ratio of fat to lean mass in addition to total body weight. Hence in most circumstances that researchers are interested in differences in weight are paralleled by differences in composition. Consider therefore the following simple example (after Speakman et al., 2002). If a 40 g mouse of strain A consisted of 30 g of lean tissue and 10 g of fat, and the lean tissue expended energy at 30 mW/g and the fat tissue expended energy at 10 mW/g, the total metabolic rate would be 1 W ($30 \times 30 + 10 \times 10$). The energy expenditure/gram of body weight would be 25 mW/g. If there was a mouse from a second strain B that had the exact same tissue metabolic rates (30 mW/g for the lean tissue and 10 mW/g for the fat tissue) but in this case the mouse weighs 50 g, comprising 30 g lean tissue and 20 g fat tissue, its total metabolism would be 1.1 W ($30 \times 30 + 20 \times 10$; 10% higher). The whole animal metabolic rate/gram of body mass would fall to 22 mW/g (12% reduced compared to strain A). Dividing by body weight in this situation therefore creates the spurious result that the metabolic rate of the heavier and fatter strain B mouse is lower, when in fact the metabolism of each of its tissues is identical to the strain A mouse. One may equally imagine a situation where the energy metabolism of the lean tissue in the lighter mouse (strain A) was 33.3 mW/g and that in the larger mouse (strain B) was 30 mW/g (an 11% lower metabolic rate), but in this situation dividing by weight would result in no difference between the two mice. Dividing by weight may therefore create spurious effects or alternatively mask real effects, but will almost never give the correct answer (Packard and Boardman, 1987; Allison et al., 1995; Poehlman and Toth, 1995; Himms Hagen, 1997; Arch et al., 2006; Butler and Kozak, 2010; Kaiyala and Schwartz, 2011). A graphical illustration of the problem is shown in **Figure 11**.

Recognizing that the intercept of the relation between energy expenditure and mass is seldom zero a different approach has been to divide by mass raised to some power <1.0 and >0.0. The value of choice differs between studies (commonly used powers are 0.75 and 0.66 (10 and 5% of studies reviewed by Tschoep et al., 2012, although occasionally other values are used – e.g., 0.83 Austad and Kristan, 2003). The source of these values are the fitted scaling exponents for the relation between mass and energy expenditure across species (Kleiber, 1961; White and Seymour, 2004, 2005). The assumption here is that changes in body composition across species as one moves from mice to elephants are similar to those as one moves from a small mouse to a larger one. This is unlikely to be the case. Nevertheless such an approach may occasionally by chance hit on the correct answer, if the inter-specific and intra-specific gradients coincide. It is, however, largely a chance effect. Sometimes it will be correct and other times not, and in yet other cases it will generate a result when none exists, in the same way dividing by mass alone can do as illustrated above. The problem is we never know which case we are dealing with. In the example above if we employ the commonly used inter-specific scaling exponents, then metabolism divided by mass raised to the 0.75 power results in a metabolic rate of 62.9 $kJ/g^{0.75}$ for strain A and 58.5 $kJ/g^{0.75}$ for strain B (a decrease of 7%). Using the other commonly used scaling exponent of 0.66 gives values of 87.6 $kJ/g^{0.66}$ for strain A and 83.2 $kJ/g^{0.66}$ for strain B (a decrease of 5%).

There is however an accepted statistical solution to this issue called analysis of covariance (ANCOVA). In effect what ANCOVA does is rather than assume a gradient for the relationship between

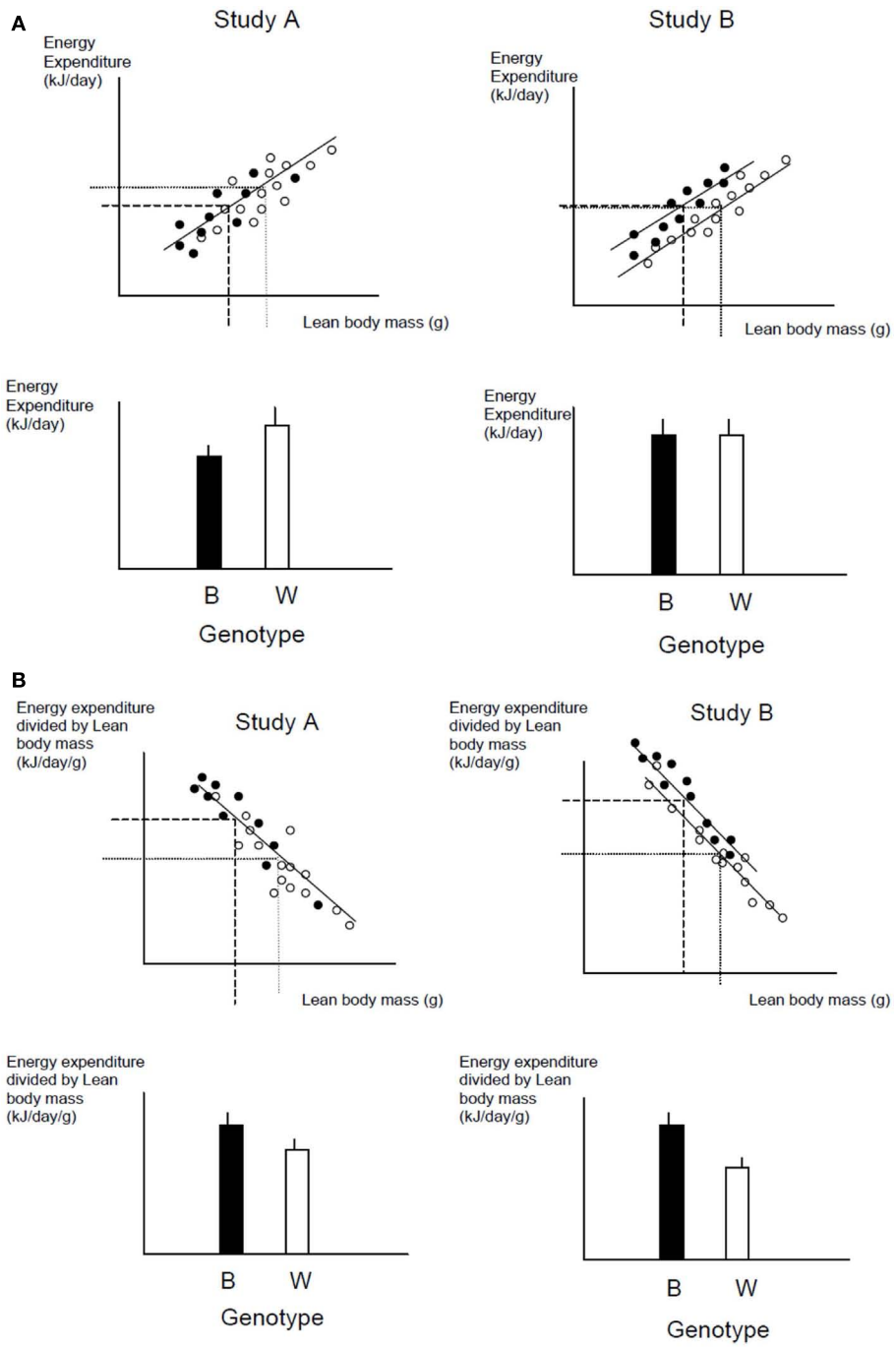


FIGURE 11 | (A) Hypothetical data from two studies of two different genotypes (black and white). In both studies there is a lean body mass difference between the two genotypes. In study **(A)**, however, the data for energy expenditure lie on a common line in relation to lean body mass. There is no difference in their energy expenditure apart from an effect due to lean body mass. In study **(B)**, the data for expenditure lie on two separate lines relative to lean body mass. In this situation, there is an effect of the genotype on expenditure independent of any mass effect. The challenge is to find an analysis that separates these two situations. If we use the raw data and average across the individuals for each genotype, the results shown below the plots as histograms reveals that there is a significant difference in study **(A)**, with energy expenditure of the black genotype being lower than that of the white one, whereas in study **(B)**, there is no significant difference in energy expenditure between the genotypes. To see if there is a genotype effect on expenditure independent of any effect of lean body mass we may divide the energy expenditure by BW **(B)**. The result in study **(B)** now reveals that expenditure in the black genotype is higher than that in the white genotype. However, this division also reveals a significant effect in study **(A)**, where none actually exists. The problem is that the division by lean mass overcompensates for the mass effect.

mass and energy demand, it fits a gradient to all the actual data. It then uses this individually tailored gradient to remove the effects of mass, and asks if there is any remaining effect of the genotype. How it does this is really simple. First a gradient is fitted to the data using regression techniques. A vertical distance is then calculated from each data point to the fitted line. These values are called the residuals to the fitted regression. The residuals are then compared between the two genotypes, taking into account the degree of freedom that is used for fitting the regression gradient to the data. An example of this process using the hypothetical data set in **Figure 11** is shown in **Figure 12** (from Tschoep et al., 2012). This is an extremely powerful solution because it makes no assumptions about the gradient but rather fits an empirical gradient to each data set. Unfortunately this approach has been used in <2% of the studies where an effect of genotype on metabolism has been examined (Tschoep et al., 2012). Examples of the use of this method are Speakman and Racey (1991) to compare the metabolic rates of echolocating and non-echolocating bats, and in mice the studies of Meyer et al. (2004) and Claret et al. (2007).

The example in **Figure 12** also makes a different, but equally important point, and that is the problem of presenting energy expenditure data as histograms. Histograms which show raw metabolic rates (Watts), or metabolic rates divided by body mass (or by fat-free mass: see below) do not provide any information about what is happening in the relationship between metabolism and body mass (Tschoep et al., 2012). Hence they do not provide the necessary information to evaluate what is going on. A much better approach in the presentation of these effects is to show the plot of the relationship between mass and metabolism, and if necessary add a histogram of residuals to this to emphasize the significance of the genotype effect (**Figure 13**). Tschoep et al. (2012) recommended the use of raw histograms to present these types of data should be phased out.

Since the issue with differences in body mass comes about because of the change in body composition then it has been argued that perhaps a better solution is not to use ANCOVA but to simply divide the metabolic rate by the mass of the metabolizing tissue (Butler and Kozak, 2010). Several devices are available which allow the *in vivo* measurement of fat mass and fat-free mass in mice (such as DXA, MRI, and CT scanners). Since the *in vitro* metabolic studies point to a major difference in the metabolic rates of lean and fat tissue then it has been argued that dividing by the fat-free mass is a preferable alternative to ANCOVA (Butler and Kozak, 2010). It is the second most popular method for "normalizing" energy expenditure data currently in use in the literature. If it was correct that all of the metabolism was attributable to the lean tissue mass then this would indeed be a potentially valid approach. However two things undermine this claim. The first is that lean tissue

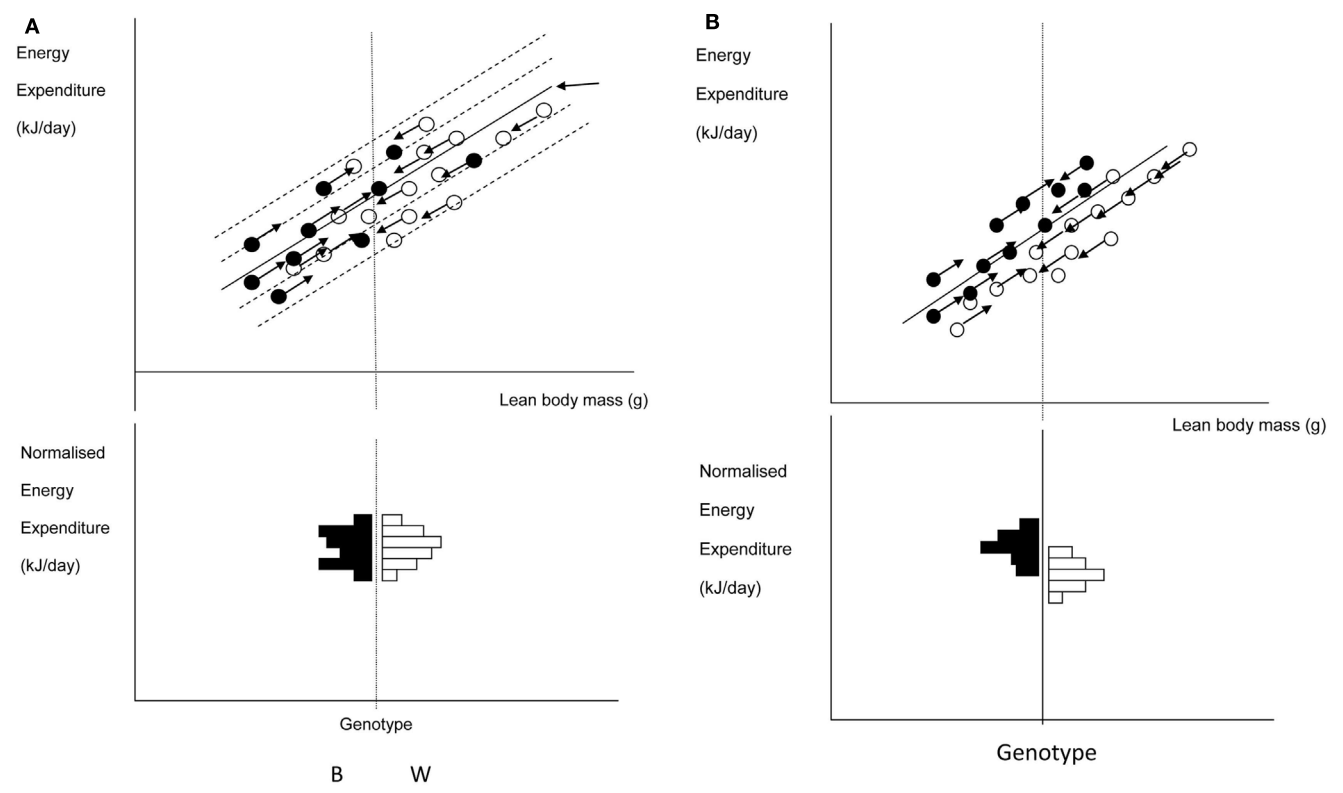


FIGURE 12 | This figure illustrates the data from the two hypothetical studies (A,B) shown in Figure 11 to illustrate the mechanism by which ANCOVA normalizes data. ANCOVA works by effectively fitting a gradient to the data and then sliding all the individual data points along imaginary parallel lines until they all group together at the average lean BW. This creates two distributions that can then be tested to see if they differ from each other. This approach is illustrated below the first panel, with the black genotype on the left of the overall mean and the white genotype on the right. As can be seen there is no significant difference. If we repeat this process for study **(B)** sliding the data down the imaginary gradients yields a different result in that the two distributions are now separated.

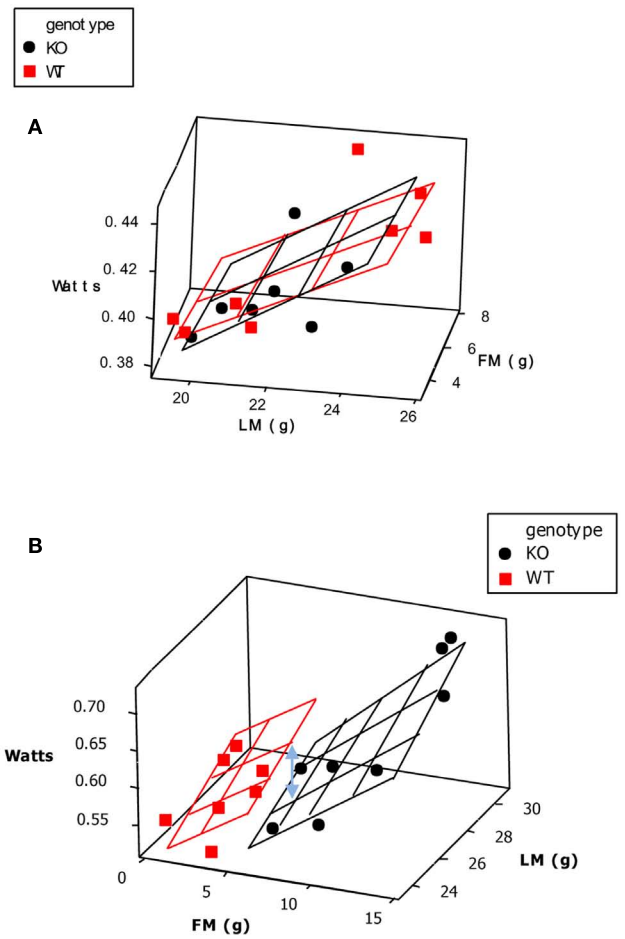


FIGURE 13 | 3D plots showing the effects of tissue specific knockouts of the p62 gene on energy expenditure taking into account the effects of lean (LM) and fat mass (FM). Wildtype mice are shown as black dots while the KO mice are shown as red squares. The black and red planes respectively represent the best fit regression models that fit the data (based on general linear modeling). In both plots the *y* variable is energy expenditure (Watts) and the *x* and *z* variables are the body component weights in grams. In **(A)** (p62 muscle specific KO) there is no difference between the planes that best describe the data using general linear modeling but in **(B)** (p62 BAT specific KO) there is a systematic reduction in the metabolism of the KO animals, illustrated as the difference between the planes (blue double headed arrow). In both cases the initial 3D plot was generated using the statistics program MINITAB and the planes were then manually added using values from the regression equations to fix their locations (from Muller et al., 2013).

is itself not homogenous, and the different components of lean tissue do not scale isometrically with total body mass, hence the relation between lean tissue mass and metabolism is also unlikely to pass through the origin – as required for a simple ratio to be used. The second problem is that while fat tissue appears to have a very low metabolism *in vitro* this does not correspond to its apparent metabolism *in vivo*. When multiple regression data are fitted to metabolic rates with lean tissue and fat tissue mass as predictors then the effect of fat tissue is often about 1/3 that of the lean tissue (Johnstone et al., 2005; Kaiyala et al., 2010). This is about 10× higher than the expectation based on *in vitro* estimates (Krebs, 1950; Elia, 1992). This is probably not because fat tissue becomes suddenly metabolically active when it is in a living body, but probably because it secretes adipokines that stimulate lean tissue metabolism. Leptin is strongly implicated in this effect (Kaiyala et al., 2010).

In the same way as dividing by mass to the power 0.66, or to the power 0.75, dividing metabolism by fat-free mass (or lean body mass: LBM) may by chance generate the correct answer. It is more likely to be the correct answer than dividing by total body mass. Nevertheless, it is also potentially the wrong answer. In the example we detailed above dividing the metabolism by lean tissue mass gives estimated metabolic rates of 33.3 mW/g LBM for strain A and 36.7 mW/g LBM for strain B – in a situation where tissue metabolic rates were actually identical. The important point is that there is no actual need to take this risk. If information is available on fat and fat-free masses of the individuals involved in the measurements then it is possible to include these two continuous variables in the ANCOVA as independent predictors of metabolism. In effect instead of fitting a simple regression model (to body weight) and calculating the residuals to this relationship, one is fitting a multiple regression model with fat and fat-free mass as predictors and then calculating the residuals to this multiple regression.

Although ANCOVA is a powerful method for analyzing these types of data it is important to recognize that it comes with its own set of assumptions and in certain circumstances will not work effectively. The first such situation worth considering is where the relationships between metabolic rate and mass (total, fat, or fat-free) are non-linear. This can normally be spotted when plotting the data as a bivariate plot and the problem can be overcome by transforming one or other of the variables. The second problem is also easily recognized from the mass-metabolism plot but is less easily overcome. This is the situation where there is absolutely no overlap in the data from the two genotypes on the mass axis. In this circumstance the variance explained by fitting two lines through the data, as is performed by ANCOVA, does not generate a significantly lower residual variance that fitting a single gradient through both data sets, leading to a non-significant genotype effect – but this may be an artifact of the data not overlapping. A solution is to calculate the regression parameters within each data set independently and then manually adjust the data using these relationships to a body mass mid way between the data sets and make the comparison at the position using a *t*-test. However, this requires extrapolation of the data beyond the limits of each data set and the resultant adjusted estimates may have too large confidence intervals to be useful. Even and Nadkarni (2012) suggested a solution to this problem when the assumptions of ANCOVA are violated would be to calculate a "metabolic equivalent weight" as the lean body mass +0.2× the fat mass, based on the fact lean body mass has approximately 20% the metabolism of lean body mass *in vivo* (after Arch et al., 2006). This remains an interesting but untested suggestion. A final issue with using fat-free and fat mass as predictor variables is how to present the data. Ideally this is done as a 3D plot with metabolism as the *y*-axis and *x* and *z* axes being fat and fat-free mass respectively. Examples of such plots for a significant and a non-significant

effect of a genotype are shown in **Figure 13** (from Muller et al., 2013).

REQUIRED SAMPLE SIZE AND POWER ANALYSIS

Another argument made by Butler and Kozak (2010) regarding the superiority of dividing by Fat-free mass as opposed to using ANCOVA is that the sample size required for ANCOVA is much larger. This is a spurious argument because simply dividing by mass is easier to calculate but does not generate any greater ability to separate two sets of data. This does however raise the interesting issue of required sample sizes for such studies. Conventional inferential statistics based on probability testing are designed to minimize the risk of a type 1 error. That is wrongly inferring something is happening when it is not. This is because studies have historically been less concerned about making type 2 errors. That is failing to spot an effect when one actually exists. However, in terms of diagnosing the effect of different genes, a type 2 error is as serious as a type 1 error. Conventional probability testing only tells us half the story (the risk of a type 1 error). What we actually also need to know is how confident we can be when we say there is no effect that we are not making a type 2 error. This is done by power analysis.

When power analysis is done in advance of an experiment being performed it can be used to establish the sample size of individual mice required in each of the genotype groups to be 95% confident we are not making a type 2 error if we find no significant effect. To do this we have to decide how big an effect would be important from a biological standpoint. For studies of energy metabolism this could be quite small (i.e., about 3–5%) as we often infer small effects on energy intake or expenditure accumulated over time may ultimately cause large effects on body composition. This is the ideal way to use power analysis. However it can also be used another way and that is to do a *post hoc* power analysis. This tells us the power we have to say there really is no significant effect of the magnitude we have detected, given the variances and sample sizes of the data sets for the two genotypes.

Using power analysis can be a very sobering exercise because it reveals that virtually every study performed to date to diagnose the effect of a given gene on energy intake has been insufficiently powered to detect the small effects in energy balance that might be biologically important. Tschoep et al. (2012) calculated that for a typical study of energy intake one might need 200 individuals/genotype to have sufficient power to avoid a type 2 error when trying to detect a difference of 3–5%.

LINKING MORE DETAILED BODY COMPOSITION MEASURES TO METABOLIC RATES

Konarzewski and Diamond (1995) compared the BMR and masses of the heart, kidneys, liver, and small intestines in six strains of mice and found that although these four organs accounted for only 17% on average of the tissue mass of the mouse they accounted for 52% of the variation in BMR. These data suggest that effects of genotype on metabolism may be mediated by effects on sizes of key organs rather than on metabolism *per se*. Using ANCOVA with the body partitioned into lean and fat mass would not be able to eliminate this as a possible explanation for a significant genotype effect on energy expenditure.

To separate these organ size effects from an effect on tissue level metabolic rates it would be necessary to measure the animals and then sacrifice them to remove and weigh their organs. This has been done on relatively few occasions in mice. Two examples are Speakman and Johnson (2000) and Selman et al. (2001). In the first study we aimed to explore the links between organ size variation and variability in the metabolism of lactating mice. In the second we aimed to investigate the contribution of organ size differences to the different metabolic rates of mice that had been selected for 38 generations for high and low food intake normalized for body mass (Hastings et al., 1997). McDevitt and Speakman (1994) also used this approach to explore the basis of cold acclimation changes in BMR in voles.

There are two separate analytical approaches that can be taken with these types of data. The first is to simply extend the multiple linear regression model to include more predictors – replacing the fat and lean tissue masses with the masses of the individual dissected organs. Selman et al. (2001) used this approach and found that in addition to the empty carcass the metabolism of the high and low food intake strains was significantly positively related to four organs (the tail, liver, spleen, and heart) the dominant effect being of the liver. In fact the strain differences in resting metabolism could be completely accounted for the by the strain differences in liver size. Given that the two strains had been selected for high and low food intake differences in the size of the liver between the strains, which were then linked to the differences in metabolic rate between the stains was not surprising. However, this study illustrates that even when there are large strain (or genotype) effects on metabolic rate these do not necessarily reflect tissue level metabolic rates but may be explained by relative differences in organ sizes. As far as I am aware nobody has yet eliminated such a possible explanation for any genotype effect on metabolic rate by measuring the sizes of all the organs in the respective genotyped animals following indirect calorimetric measurements.

There are two major issues however with this approach. The first is the ratio of variables to observations (Even and Nadkarni, 2012). Mice can be dissected into a large number of distinct organs. Selman et al. (2001), for example, split their mice into 19 different components. However, they only measured 39 individual mice hence the ratio of measurements to variables was just over 2. Ideally in this type of multiple regression model the target to aim for is a ratio of above 6. The second problem is statistical inference in multiple linear regression models is only possible if the predictor variables are independent of each other. Yet organ masses are clearly correlated. Bigger individuals have the tendency for all their organs to be on the large side, and smaller animals show the opposite trend. To overcome this problem one method is to express the relationship between each organ size and the total body mass and then calculate the residual values to the fitted regression. One can also do the same for the RMR measurement and then include the residual masses of the organs into a multiple regression model as predictors and the residual RMR measurement as the dependent variable. When Selman et al. (2001) did this the effect of the liver remained highly significant (**Figure 14**), but the effects of the tail, spleen, and heart were no longer significant suggesting their effects in the previous analysis were artifacts of being correlated

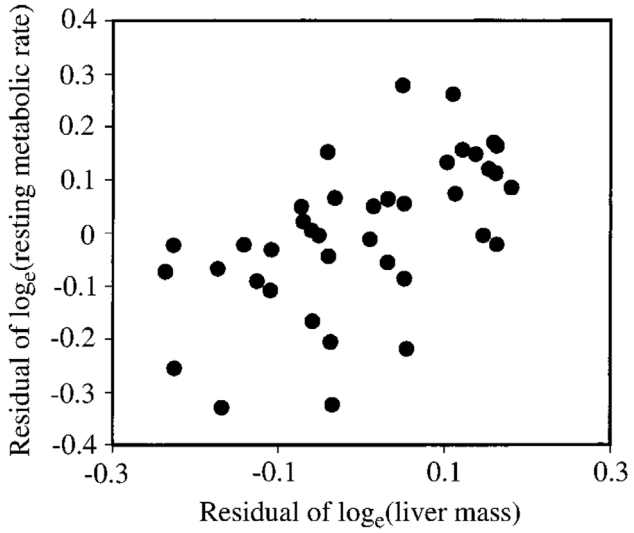


FIGURE 14 | Residual metabolic rate plotted against residual liver mass (both with the covariable effects of total body mass removed) across 39 individual mice from two strains (from Selman et al., 2001).

to the total body weight. Interestingly, however, a negative effect of pelage weight emerged in this analysis, which was not found in the original analysis.

To overcome the first problem of the ratio of measurements to variables there are two different approaches that can be employed. The first is to group different organs and tissues together in functional groups to reduce the number of variables. Selman et al. (2001) for example reduced their 19 tissues to five functional groups (making the ratio of variables to measurements about eight) and repeated the analysis. The liver again emerged as the only significant predictor. A different approach was used by Speakman and Johnson (2000) in 59 lactating mice that were dissected into 18 different organs and tissues. In this group a principal components analysis was run to compress the 18 variables into five principal components which still retained 80% of the original variance. The only scores to enter the stepwise regression with RMRt as the dependent variable were those for PC1, which was a general body size component. When residual RMRt was used even these scores were not significant.

SUMMARY

Measuring the energy metabolism of mice is a key skill that is necessary to understand the impact of genetic manipulations, or of drug and compound treatments, on energy metabolism. Generally daily energy expenditure can be partitioned into different components: basal or resting metabolism, the costs of thermoregulation and physical activity, and the thermic effect of feeding. Measuring daily energy demands is best performed using large chambers that permit the animal to replicate its home cage behavior. These larger chambers may be linked to a single analyzer or have several chambers sequentially monitored by one analyzer. Decomposing the outputs from such systems to yield the component metabolic rates is currently not feasible with the required degree of accuracy. Component metabolic rates are rather better determined using small fast washout chambers where a single chamber is linked to a single analyzer. Outputs from both types of system pose challenges for analysis, in particular how best to correct for differences in body mass and composition between individuals. Generally the most appropriate statistical approach for treatment of such data is ANCOVA.

ACKNOWLEDGMENTS

I am grateful to the authors of previously published papers who allowed me to use Figures from their work in this paper. Lobke Vaanholt kindly generated **Figure 2** from data previously collected in a study of variation of weight loss on calorie restricted diets. Rachel Sinclair collected the data in **Figure 10A** as part of her Ph.D. study. Timo Muller kindly provided the data for **Figure 10B** and Brent Wisse via John Lighton kindly provided the data in **Figure 10C**. I am grateful to Jon Arch, David Bryant, Matthias Tschoep, Paul Tatner, Henk Visser, Dirkjan Masman, Don Thomas, Ed Melanson, John Lighton, Patrick Even, Eric Ravussin, Ron Khan, Ela Krol, DeHua Wang, Andrew Butler, Alex Johnstone, Diane Jackson, Paul Trayhurn, Lobke Vaanholt, Catherine Hambly, Luiza Duarte, Colin Selman, Lutz Bunger, and Bill Hill for valuable discussions about respirometry methods and analysis. Two anonymous referees made many valuable suggestions to improve the paper.

REFERENCES

Allison, D. B., Paultre, F., Goran, M. I., Poehlman, E. T., and Heymsfield, S. B. (1995). Statistical considerations regarding the use of ratios to adjust data. *Int. J. Obes.* 19, 644–652.

Arch, J. R. S., Hislop, D., Wang, S. J. Y., and Speakman, J. R. (2006). Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals. *Int. J. Obes.* 30, 1322–1331.

Aschoff, J., and Phol, H. (1970). Der ruheumsatz von vogeln als funktion der tageszeit und der Korpergrosse. *J. Ornithol.* 111, 38–47.

Austad, S. N., and Kristan, D. M. (2003). Are mice calorically restricted in nature? *Aging Cell* 2, 201–207.

Bal, N. C., Maurya, S. K., Sopariwala, D. H., Sahoo, S. K., Gupta, S. C., Shaikh, S. A., et al. (2012). Sarcolipin is a newly identified regulator of muscle-based thermogenesis in mammals. *Nat. Med.* 18, 1575–1579.

Bartholomew, G. A., Vleck, D., and Vleck, C. M. (1981). Instantaneous measurements of oxygen consumption during pre-flight warm-up and post-flight cooling in Sphingid and saturniid moths. *J. Exp. Biol.* 90, 17–32.

Bartolomucci, A., Cabassi, A., Govoni, P., Ceresini, G., Cero, C., and Berra, D., et al. (2009). Metabolic consequences and vulnerability to diet-induced obesity in male mice under chronic social stress. *PLoS ONE* 4:e4331. doi:10.1371/journal.pone.0004331

Bech, C., and Praesteng, K. E. (2004). The thermoregulatory use of heat increment of feeding in the tawny owl (Strix aluco). *J. Therm. Biol.* 29, 649–654.

Bjursell, M., Gerdin, A. K., Lelliott, C. J., Egecioglu, E., Elmgren, A., Törnell, J., et al. (2008). Acutely reduced locomotor activity is a major contributor to Western diet induced obesity in mice. *Am. J. Physiol.* 294, E251–E260.

Boratynski, Z., and Koteja, P. (2009). The association between body mass, metabolic rates and survival of bank voles. *Funct. Ecol.* 23, 330–339.

Bruinzeel, L. W., and Piersma, T. (1998). Cost reduction in the cold: heat generated by terrestrial locomotion partially substitutes for thermoregulation costs in knot Calidris canutus. *Ibis* 140, 323–328.

Bursztein, S., Saphar, P., Singer, P., and Elwyn, D. H. (1989). A mathematical analysis of indirect calorimetry measurements in acutely ill patients. *Am. J. Clin. Nutr.* 50, 227–230.

Butler, A. A., and Kozak, L. P. (2010). A recurring problem with the analysis of energy expenditure in genetic models expressing lean, and obese phenotypes. *Diabetes* 59, 323–329.

Butler, P. J., Green, J. A., Boyd, I. L., and Speakman, J. R. (2004). Measuring metabolic rate in the field: the pros and cons of the doubly-labelled water and heart rate methods. *Funct. Ecol.* 18, 168–183.

Cannon, B., and Nedergaard, J. (2009). Thermogenesis challenges the adipostat hypothesis for body-weight control. *Proc. Nutr. Soc.* 68, 401–407.

Cannon, B., and Nedergaard, J. (2011). Nonshivering thermogenesis and its adequate measurement in metabolic studies. *J. Exp. Biol.* 214, 242–253.

Claret, M., Smith, M. A., Batterham, R. L., Selman, C., Choudhury, A. I., Fryer, L. G. D., et al. (2007). AMPK is essential for energy homeostasis regulation and glucose sensing by POMC and AgRP neurons. *J. Clin. Invest.* 117, 2325–2336.

Davis, J. E., and van Dyke, H. B. (1932). The measurement of oxygen consumption of small animals. *J. Biol. Chem.* 95, 73–78.

Davis, J. E., and van Dyke, H. B. (1933). The oxygen consumption of fasting white mice. *J. Biol. Chem.* 100, 455–462.

Deveaux, V., Cadoucal, T., Ichigotani, Y., Teixeira-Clerc, F., Louvet, A., Manin, S., et al. (2009). Cannabinoid CB2 receptor potentiates obesity associated inflammation, insulin resistance and hepatic steatosis. *PLoS ONE* 4:e5844. doi:10.1371/journal.pone.0005844

Duarte, L., Vaanholt, L., Sinclair, R., Gamo, Y., and Speakman, J. R. (2010). Limits to sustained energy intake XII: is the poor relationship between RMR and reproductive performance because RMR is not a repeatable trait? *J. Exp. Biol.* 213, 278–287.

Elia, M. (1992). "Organ and tissue contribution to metabolic rate," in *Energy Metabolism: Tissue Determinants and Cellular Corollaries,* eds M. Elia, J. M. Kinney, and H. N. Tucker (New York: Raven), 61–80.

Elia, M., and Livesey, G. (1992). Energy expenditure and fuel selection in biological systems: the theory and practice of calculations based on indirect calorimetry and tracer methods. *World Rev. Nutr. Diet* 70, 68–131.

Enerbäck, S., Jacobsson, A., Simpson, E. M., Guerra, C., Yamashita, H., Harper, M. E., et al. (1997). Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. *Nature* 387, 90–94.

Even, P. C., Mokhtarian, A., and Pele, A. (1994). Practical aspects of indirect calorimetry in laboratory animals. *Neurosci. Biobehav. Rev.* 18, 435–477.

Even, P. C., and Nadkarni, N. A. (2012). Indirect calorimetry in laboratory mice and rats: principles, practical considerations, interpretation and perspectives. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 303, R459–R476.

Even, P. C., Perrier, E., Aucouturier, J. L., and Nicolaidis, S. (1991). Utilisation of the method of Kalman filtering for performing the online computation of background metabolism in the free-moving free-feeding rat. *Physiol. Behav.* 49, 177–187.

Feldmann, H. M., Golozoubova, V., Cannon, B., and Nedergaard, J. (2009). UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell Metab.* 9, 203–209.

Ferrannini, E. (1988). The theoretical basis of indirect calorimetry – a review. *Metab. Clin. Exp.* 37, 287–301.

Fletcher, Q. E., Selman, C., Boutin, S., McAdam, A. G., Woods, S. B., Seo, A. Y., et al. (2013). Oxidative damage increases with reproductive energy expenditure and is reduced by food supplementation. *Evolution* (in press).

Fuhrman, G. J., Mclin, E. D., and Turner, M. L. (1946). The effect of time of day on the metabolic rate of albino mice – a manometric method. *Am. J. Physiol.* 147, 284–288.

Gallivan, G. J. (1992). What are the metabolic rates of cetaceans? *Physiol. Zool.* 65, 1285–1287.

Gaskill, B. N., Gordon, C. J., Pajor, E. A., Lucas, J. R., Davis, J. K., and Garber, J. P. (2012). Heat or insulation: behavioural titration of mouse preference for warmth or access to a nest. *PLoS ONE* 7:e32799 doi:10.1371/journal.pone.0032799

Gaskill, B. N., Rohr, S. A., Pajor, E. A., Lucas, J. R., and Garner, J. P. (2009). Some like it hot: mouse temperature preferences in laboratory housing. *Appl. Anim. Behav. Sci.* 116, 279–285.

Golozoubova, V., Cannon, B., and Nedergaard, J. (2006). UCP-1 is essential for adaptive adrenergic nonshivering thermogenesis. *Am. J. Physiol. Endocrinol. Metab.* 291, E350–E357.

Golozoubova, V., Gullberg, H., Matthias, A., Cannon, B., Vennström, B., and Nedergaard, J. (2004). Depressed thermogenesis but competent brown adipose tissue recruitment in mice devoid of all hormone-binding thyroid hormone receptors. *Mol. Endocrinol.* 18, 384–401.

Golozoubova, V., Hohtola, E., Matthias, A., Jacobsson, A., Cannon, B., and Nedergaard, J. (2001). Only YCP-1 can mediate adaptive non-shivering thermogenesis in the cold. *FASEB J.* 15, 2048–2055.

Gordon, C. J. (1993). *Temperature Regulation of Laboratory Rodents.* Cambridge: Cambridge University Press.

Gordon, C. J., Becker, P., and Ali, J. S. (1998). Behavioral thermoregulatory responses of single- and group-housed mice. *Physiol. Behav.* 65, 255–262.

Haldane, J. S. (1912). *Methods of Air Analysis.* London: Griffin.

Hall, K. H., Heymsfield, S., Kemnitz, J., Klein, S., Schoeller, D. A., and Speakman, J. R. (2012). Energy balance, and body weight regulation: a useful concept for understanding the obesity epidemic. *Am. J. Clin. Nutr.* 95, 989–994.

Hambly, C., Harper, E. J., and Speakman, J. R. (2002). The cost of flight in the zebra finch (Taenyopygia guttata): a novel approach based on elimination of carbon-13 labelled bicarbonate. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 172, 529–539.

Hambly, C., Pinshow, B., Wiersma, P., Verhulst, S., Piertney, S. B., Harper, E. J., et al. (2004a). Comparison of the cost of short flights in a nectarivorous and non-nectarivorous bird. *J. Exp. Biol.* 207, 3959–3968.

Hambly, C., Harper, E. J., and Speakman, J. R. (2004b). The energy cost of loaded flight is substantially lower than expected due to alterations in flight kinematics. *J. Exp. Biol.* 207, 3969–3976.

Hambly, C., and Voigt, C. C. (2011). Measuring energy expenditure in birds using bolus injections of C-13-labelled Na bicarbonate. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 158, 323–328.

Hastings, I. M., Moruppa, S. M., Bunger, L., and Hill, W. G. (1997). Effects of selection on food intake in the adult mouse. *J. Anim. Breed. Genet.* 114, 419–433.

Hayes, J. P., Speakman, J. R., and Racey, P. A. (1992). Sampling bias in respirometry. *Physiol. Zool.* 65, 604–619.

Hays, G. C., Webb, P. I., French, J., and Speakman, J. R. (1990). Doppler radar – a noninvasive technique for measuring ventilation rate in resting bats. *J. Exp. Biol.* 150, 443–447.

Heldmaier, G. (1971). Non-shivering thermogenesis and body size in mammals. *Z. Vgl. Physiol.* 73, 222–231.

Himms Hagen, J. (1997). On raising energy expenditure in ob/ob mice. *Science* 276, 1132–1133.

Hoggard, N., Rayner, D. V., Johnston, S. L., and Speakman, J. R. (2004). Peripherally administered [Nle4, D-Phe7]-αMSH increases resting metabolic rate, while peripheral AgRP has no effect, in wild type C57BL/6 and ob/ob mice. *J. Mol. Endocrinol.* 33, 693–703.

Humphries, M. M., and Careau, V. (2011). Heat for nothing or activity for free? Evidence and implications of activity thermoregulatory heat substitution. *Integr. Comp. Biol.* 51, 419–431.

Hussein, H. K. (1991). Effect of temperature and body size on the metabolic rate of Egyptian house mice (Mus musculus) and the roof rat (Rattus rattus). *J. Islamic Acad. Sci.* 4, 249–252.

Jackson, D. M., Hambly, C., Trayhurn, P., and Speakman, J. R. (2001a). Can non-shivering thermogenesis in brown adipose tissue following NA injection be quantified by changes in overlying surface temperatures using infrared thermography? *J. Therm. Biol.* 26, 85–93.

Jackson, D. M., Trayhurn, P., and Speakman, J. R. (2001b). Associations between energetics and over-winter survival in the short-tailed field vole Microtus agrestis. *J. Anim. Ecol.* 70, 633–640.

Jansky, L. (1973). Non-shivering thermogenesis and its thermoregulatory significance. *Biol. Rev. Camb. Philos. Soc.* 48, 85–132.

Johnson, M. S., and Speakman, J. R. (2001). Limits to sustained energy intake V: effect of cold exposure during lactation in Mus musculus. *J. Exp. Biol.* 204, 1967–1977.

Johnson, M. S., Thomson, S. C., and Speakman, J. R. (2001). Limits to sustained energy intake I. Lactation in the laboratory mouse Mus musculus. *J. Exp. Biol.* 204, 1925–1935.

Johnstone, A. M., Murison, S. D., Duncan, J. S., Rance, K. A., and Speakman, J. R. (2005). Factors influencing variation in basal metabolic rate include fat-free mass, fat mass, age, and circulating thyroxine but not sex, circulating leptin, or triiodothyronine. *Am. J. Clin. Nutr.* 82, 941–948.

Kaiyala, K. J., Morton, G. J., Leroux, B. G., Ogimoto, K., Wisse, B., and Schwartz, M. W. (2010). Identification of body fat mass as a major determinant of metabolic rate in mice. *Diabetes* 59, 1657–1666.

Kaiyala, K. J., and Ramsay, D. S. (2011). Direct animal calorimetry, the underused gold standard for quantifying the fire of life. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 158, 252–262.

Kaiyala, K. J., and Schwartz, M. W. (2011). Toward a more complete (and less controversial) understanding of energy expenditure and its role in obesity pathogenesis. *Diabetes* 60, 17–23.

Karp, C. L. (2012). Unstressing intemperate models: how cold stress undermines mouse modelling. *J. Exp. Med.* 209, 1069–1074.

Kleiber, M. (1961). *The Fire of Life: An Introduction to Animal Energetics.* New York: Wiley.

Konarzewski, M. K., and Diamond, J. (1995). Evolution of metabolic rate and organ masses in laboratory mice. *Evolution* 49, 1239–1248.

Koteja, P. (1996). Measuring energy metabolism with open-flow respirometric systems: which design to choose? *Funct. Ecol.* 10, 675–677.

Krebs, H. A. (1950). Body size and tissue respiration. *Biochim. Biophys. Acta* 4, 249–269.

Krol, E., Johnson, M. S., and Speakman, J. R. (2003). Limits to sustained energy intake VIII. Resting metabolic rate and organ morphology of laboratory mice lactating at thermoneutrality. *J. Exp. Biol.* 206, 4283–4291.

Krol, E., Murphy, M., and Speakman, J. R. (2007). Limits to sustained energy intake X: effects of fur removal on reproductive performance in laboratory mice. *J. Exp. Biol.* 207, 4233–4243.

Krol, E., and Speakman, J. R. (2003a). Limits to sustained energy intake VII. Milk energy output in laboratory mice at thermoneutrality. *J. Exp. Biol.* 206, 4267–4281.

Krol, E., and Speakman, J. R. (2003b). Limits to sustained energy intake VI. Energetics of lactation in laboratory mice at thermoneutrality. *J. Exp. Biol.* 206, 4255–4266.

Ksiazek, A., Konarzewski, M. K., and Lapo, I. B. (2004). Anatomic and energetic correlates of divergent selection for basal metabolic rate in laboratory mice. *Physiol. Zool.* 77, 890–899.

Labocha, M. K., Sadowska, E. T., Balinga, K., Semer, A., and Koteja, P. (2004). Individual variation and repeatability of basal metabolism in the bank vole, Clethrionomys glareolus. *Proc. R. Soc. Lond. B Biol. Sci.* 271, 367–372.

Lifson, N., Gordon, G. B., and McClintock, R. (1955). Measurement of total carbon dioxide production by D2O18. *J. Appl. Physiol.* 7, 704–710.

Lighton, J. R. B. (2008). *Measuring Metabolic Rates: A Manual for Scientists.* Oxford: Oxford University Press.

Liu, X. T., Rossmeisl, M., McClaine, J., and Kozak, L. P. (2003). Paradoxical resistance to diet-induced obesity in UCP-1 deficient mice. *J. Clin. Invest.* 111, 399–407.

Lodhi, I. J., and Semenkovich, C. F. (2009). Why we should put clothes on mice. *Cell Metab.* 9, 111–112.

McDevitt, R. M., and Speakman, J. R. (1994). Central limits to sustainable metabolic rate has no role in cold acclimation of the short-tailed field vole (Microtus agrestis). *Physiol. Zool.* 67, 1117–1139.

McNab, B. K. (1997). On the utility of uniformity in the definition of basal metabolism. *Physiol. Zool.* 70, 718–720.

Meyer, C. W., Klingenspor, M., Rozman, J., and Heldmaier, G. (2004). Gene or size: metabolic rate and body temperature in obese growth hormone-deficient dwarf mice. *Obes. Res.* 12, 1509–1518.

Muller, T. D., Lee, S. J., Jastroch, M., Stemmer, K., Aichler, M., Abplanalp, B., Anathakrishna, G., et al. (2013). p62 links beta-adrenergic input with mitochondrial function. *J. Clin. Invest.* 123, 469–478.

Nonogaki, K., Abdallah, L., Goulding, E. H., Bonasera, S. J., and Tecott, L. H. (2003). Hyperactivity and reduced energy cost of physical activity in serotonin 5-HT2c receptor mutant mice. *Diabetes* 52, 315–320.

Overton, J. M. (2010). Phenotyping small animals as models for the human metabolic syndrome: thermoneutrality matters. *Int. J. Obes.* 34, S53–S58.

Packard, G. C., and Boardman, T. J. (1987). "The mis-use of ratios to scale physiological data that vary allometrically with body size," in *New Directions in Ecological Physiology*, eds M. E. Feder, A. F. Bennett, W. W. Burggren, and R. B. Huey (Cambridge: University press Cambridge), 216–239.

Peltonen, L., and McKusick, V. A. (2001). Genomics and medicine – dissecting human disease in the postgenomic era. *Science* 291, 1224–1229.

Pertwee, R. G., and Tavendale, L. (1977). The effects of δ 9-tetrahydrocannabinol on the rates of oxygen consumption of mice. *Br. J. Pharmacol.* 60, 559–568.

Pincede, I., Pollin, B., Meert, T., Plaghki, L., and Le Bars, D. (2012). Pschophysics of a nociceptive test in the mouse: ambient temperature as a key factor for variation. *PLoS ONE* 7:e36699. doi:10.1371/journal.pone.0036699

Poehlman, E. T., and Toth, M. J. (1995). Mathematical ratios lead to spurious conclusions regarding age-related and sex-related differences in resting metabolic-rate. *Am. J. Clin. Nutr.* 61, 482–485.

Russell, G. A., and Chappell, M. A. (2007). Is BR repeatable in deer mice? Organ mass correlates wand the effects of cold acclimation and natal altitude. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 177, 75–87.

Sadowska, E. T., Baliga-klimczyk, K., Labochha, M. K., and Koteja, P. (2009). Genetic correlations in a wild rodent: grass eaters and fast-growers evolve high basal metabolic rates. *Evolution* 63, 1530–1539.

Schefer, V., and Talan, M. I. (1996). Oxygen consumption in adult and aged C57BL/6 mice during acute treadmill exercise of different intensity. *Exp. Gerontol.* 31, 387–392.

Scholander, P. F., Hock, R., Walters, V., Johnson, F., and Irving, L. (1950). Heat regulation in some arctic and tropical mammals and birds. *Biol. Bull.* 99, 237–258.

Selman, C., Lumsden, S., Bunger, L., Hill, W. G., and Speakman, J. R. (2001). Resting metabolic rate and morphology in mice (Mus musculus) selected for high and low food intake. *J. Exp. Biol.* 204, 777–784.

Simons, M. J. P., Reimert, I., van der Vinne, V., Hambly, C., Vaanholt, L. M., Speakman, J. R., et al. (2011). Ambient temperature shapes reproductive output during pregnancy and lactation in the common vole (Microtus arvalis): a test of the heat dissipation limit theory. *J. Exp. Biol.* 214, 38–49.

Simonson, D. C., and deFronzo, R. A. (1990). Indirect calorimetry – methodological and interpretative problems. *Am. J. Physiol.* 258, E399–E412.

Speakman, J. R. (1997). *Doubly-Labelled Water: Theory and Practice.* Berlin: Springer.

Speakman, J. R. (2000). The cost of living: Field metabolic rates of small mammals. *Adv. Ecol. Res.* 30, 177–297.

Speakman, J. R., Anderson, M. E., and Racey, P. A. (1989). The energy-cost of echolocation in pipistrelle bats (*Pipistrellus-pipistrellus*). *J. Comp. Physiol. A* 165, 679–685.

Speakman, J. R., Fletcher, Q. E., and Vaanholt, L. M. (2013). The "39 steps": an algorithm for performing statistical analysis of energy intake and expenditure data. *Dis. Model. Mech.* 6, (in press).

Speakman, J. R., Hambly, C., Mitchell, S. E., and Krol, E. (2008). The contribution of animal models to the study of obesity. *Lab. Anim.* 42, 413–432.

Speakman, J. R., and Keijer, J. (2013). Not so hot. Optimal housing temperatures for mice to mimic the thermal environment of humans. *Mol. Metab.*

Speakman, J. R., and Krol, E. (2005). Validation of the doubly-labelled water method in a small mammal. *Physiol. Biochem. Zool.* 78, 650–667.

Speakman, J. R., Krol, E., and Johnston, M. S. (2004). The functional significance of individual variations in BMR. *Physiol. Biochem. Zool.* 77, 900–915.

Speakman, J. R., McDevitt, R. M., and Cole, K. R. (1993). Measurement of basal metabolic rates – don't lose sight of reality in the quest for comparability. *Physiol. Zool.* 66, 1045–1049.

Speakman, J. R., Racey, P. A., Haim, A., Webb, P. I., Ellison, G. T. H., and Skinner, J. D. (1994). Interindividual and intraindividual variation in daily energy expenditure of the puhed mouse (Saccostomus campestris). *Funct. Ecol.* 8, 336–342.

Speakman, J. R., and Racey, P. A. (1991). No cost of echolocation for bats in flight. *Nature* 350, 421–423.

Speakman, J. R., and Rossi, F. P. (1999). No support for socio-physiological suppression effect on metabolism of paired white mice (Mus sp.). *Funct. Ecol.* 13, 373–382.

Speakman, J. R., Selman, C., McLaren, J. S., and Harper, J. E. (2002). Living fast, dying when? The links between energetics and ageing. *J. Nutr.* 132, 1583S–1597S.

Speakman, J. R., and Thomson, S. C. (1997). Validation of the labeled bicarbonate technique for measurement of short term energy expenditure in the mouse. *Z. Ernahrungswiss.* 36, 273–277.

Speakman, J. R., and Johnson, M. S. (2000). "Relationships between

resting metabolic rate and morphology in lactating mice: what tissues are the major contributors to resting metabolism?" in *"Living in the Cold: vol III,"* eds G. Heldmaier and M. Klingenspor (Berlin: Springer), 479–486.

Swoap, S. J., Li, C., Wess, J., Parsons, A. D., Williams, T. D., and Overton, J. M. (2008). Vagal tone dominates autonomic control of mouse heart rate at thermoneutrality. *Am. J. Physiol. Heart Circ. Physiol.* 294, H1581–H1588.

Thomas, D. W., Cloutier, D., and Gagne, D. (1990). Arrythmic breathing, apnea and non-steady state oxygen uptake in hibernating little brown bats. *J. Exp. Biol.* 149, 395–406.

Tschoep, M. H., Speakman, J. R., Arch, J. R. S., Auwerx, J., Brüning, J. C., Chan, L., et al. (2012). A guide to analysis of mouse energy metabolism. *Nat. Methods* 9, 57–63.

Vaanholt, L. M., Magee, V., and Speakman, J. R. (2012). Factors predicting individual variability on diet induced weight loss in MF1 mice. *Obesity (Silver Spring)* 20, 285–294.

Valle, A., Hoggard, N., Adams, A. C., Roca, P., and Speakman, J. R. (2008). Chronic central administration of apelin-13 over ten days increases food intake, body weight, locomotor activity and body temperature in C57BL/6 mice. *J. Neuroendocrinol.* 20, 79–84.

van Klinken, J. B., van den Berg, S. A. A., Havekes, L. M., and van Dijk, K. W. (2012). Estimation of activity related energy expenditure and resting metabolic rate in freely moving mice from indirect calorimetry data. *PLoS ONE* 7:e36162. doi:10.1371/journal.pone.0036162

Virtue, S., Even, P., and Vidal-Puig, A. (2012). Below thermoneutrality, changes in activity do not drive changes in total daily energy expenditure between groups of mice. *Cell Metab.* 16, 665–671.

Voigt, C. C., and Holderied, M. W. (2012). High Manoeuvring costs force narrow winged molossid bats to forage in open space. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 182, 415–424.

Voigt, C. C., and Lewanzik, D. (2012). "No cost of echolocation for flying bats" revisited. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 182, 831–840.

Walsberg, G. E., and Hoffman, T. C. M. (2005). Direct calorimetry reveals large errors in respirometric estimates of energy expenditure. *J. Exp. Biol.* 208, 1035–1043.

Weir, J. B. (1949). New methods for calculating metabolic rate with special reference to protein metabolism. *J. Physiol. (Lond.)* 109, 1–9.

White, C. R., and Seymour, R. S. (2004). Mammalian basal metabolic rate is proportional to body mass (2/3). *Proc. Natl. Acad. Sci. U.S.A.* 100, 4046–4049.

White, C. R., and Seymour, R. S. (2005). Allometric scaling of mammalian metabolism. *J. Exp. Biol.* 208, 1611–1619.

Wu, S. H., Zhang, L., Speakman, J. R., and Wang, D. H. (2009). Limits to sustained energy intake XI: a test of the heat dissipation limit hypothesis in lactating Brandt's voles (Lasiopodomys brandtii). *J. Exp. Biol.* 212, 3455–3465.

Zerba, E., and Walsberg, G. E. (1992). Exercise generated heat contributes to thermoregulation by Gambel quail in the cold. *J. Exp. Biol.* 171, 409–422.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 09 November 2012; paper pending published: 27 November 2012; accepted: 12 February 2013; published online: 14 March 2013.

Citation: Speakman JR (2013) Measuring energy metabolism in the mouse – theoretical, practical, and analytical considerations. Front. Physiol. ***4****:34. doi: 10.3389/fphys.2013.00034*

This article was submitted to Frontiers in Integrative Physiology, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

METHODS ARTICLE
published: 25 March 2013
doi: 10.3389/fphys.2013.00047

Issues in characterizing resting energy expenditure in obesity and after weight loss

Anja Bosy-Westphal[1,2]†, Wiebke Braun[1], Britta Schautz[1] and Manfred J. Müller[1]*

[1] Institut für Humanernährung und Lebensmittelkunde, Christian-Albrechts-Universität zu Kiel, Kiel, Germany
[2] Institut für Ernährungsmedizin, Universität Hohenheim, Stuttgart, Germany

Edited by:
Patrick C. Even, AgroParisTech, France

Reviewed by:
Abdul G. Dulloo, University of Fribourg, Switzerland
Patrick C. Even, AgroParisTech, France

****Correspondence:***
Anja Bosy-Westphal, Institut für Humanernährung und Lebensmittelkunde, Christian-Albrechts-Universität zu Kiel, Düsternbrooker Weg 17, 24105 Kiel, Germany.
e-mail: anja.bosy-westphal@uni-hohenheim.de

† Present address:
Anja Bosy-Westphal, Institut für Ernährungsmedizin (180c), Universität Hohenheim, Fg. Angewandte Ernährungswissenschaft/Diätetik, Fruwirthstraße 12, 70599 Stuttgart, Germany.

Limitations of current methods: Normalization of resting energy expenditure (REE) for body composition using the 2-compartment model fat mass (FM), and fat-free mass (FFM) has inherent limitations for the interpretation of REE and may lead to erroneous conclusions when comparing people with a wide range of adiposity as well as before and after substantial weight loss.

Experimental objectives: We compared different methods of REE normalization: (1) for FFM and FM (2) by the inclusion of %FM as a measure of adiposity and (3) based on organ and tissue masses. Results were compared between healthy subjects with different degrees of adiposity as well as within subject before and after weight loss.

Results: Normalizing REE from an "REE vs. FFM and FM equation" that (1) was derived in obese participants and applied to lean people or (2) was derived before weight loss and applied after weight loss leads to the erroneous conclusion of a lower metabolic rate (i) in lean persons and (ii) after weight loss. This is revealed by the normalization of REE for organ and tissue masses that was not significantly different between lean and obese or between baseline and after weight loss. There is evidence for an increasing specific metabolic rate of FFM with increasing %FM that could be explained by a higher contribution of liver, kidney and heart mass to FFM in obesity. Using "REE vs. FFM and FM equations" specific for different levels of adiposity (%FM) eliminated differences in REE before and after weight loss in women.

Conclusion: The most established method for normalization of REE based on FFM and FM may lead to spurious conclusions about metabolic rate in obesity and the phenomenon of weight loss-associated adaptive thermogenesis. Using %FM-specific REE prediction from FFM and FM in kg may improve the normalization of REE when subjects with wide differences in %FM are investigated.

Keywords: resting energy expenditure, normalization, fat free mass, fat mass, organ mass, obesity weight loss, adaptive thermogenesis

INTRODUCTION

In order to differentiate phenotypes of high and low metabolic rate or to understand the changes in resting energy expenditure (REE) that accompany weight loss or gain normalization of REE is required (Arch et al., 2006; Kaiyala and Schwartz, 2011). Larger people naturally have a higher REE than smaller people. However, using body mass as a parameter for REE, normalization would lead to the errant conclusion that obese people have a lower REE than lean individuals. Because in obese people body mass consists of a higher proportion of metabolically inert fat mass (FM), the specific metabolic rate (i.e., energy expenditure per unit body mass) is lower. Body composition is therefore a crucial determinant of REE.

Owing to the widespread use of the 2-compartment model of body composition, normalization of REE is generally performed by accounting for metabolically active fat-free mass (FFM). FFM is the main determinant of REE explaining between 53 and 88% (usually not more than 75%) of its variance (reviewed by Nelson et al., 1992). REE scales with FFM, the regression line has a significant intercept term of about 400 kcal/day (Müller et al., 2011; Heymsfield et al., 2012). Numerous regression equations have been published for prediction of REE from FFM (reviewed by Wang et al., 2000). These equations all have similar slopes varying from 19.7 to 24.5 kcal × kg FFM^{-1} × day^{-1} and positive intercepts varying from 186 to 662 kcal/day (Wang et al., 2000). Differences in the methodology used to estimate FFM did not explain the discrepancies between those equations that may rather be due to population differences (Korth et al., 2007).

The volume and mass of individual tissues can now be measured with great accuracy and a new reference man has been proposed (Later et al., 2010). Reference data on detailed body composition of young normal weight adults are given in **Table 1**. Very recently these data have been used to explain the non-zero intercept of the REE-FFM function (Heymsfield et al., 2012). Using different models including adipose tissue free mass, adipose tissue, sex as well as individual organ masses the explained

Table 1 | Detailed body composition in a healthy adult reference population*.

	Men		Women		*P*-value
	Mean ± *SD*	Range	Mean ± *SD*	Range	
Age, years	31.6 ± 5.8	20–40	30.7 ± 5.9	20–40	0.344
Height, m	1.80 ± 0.06	1.68–1.93	1.69 ± 0.07	1.49–1.86	<0.001
Weight, kg	90.2 ± 18.9	58.2–137.1	85.3 ± 22.7	44.7–144.9	0.161
BMI, kg/m^2	27.6 ± 5.2	19.0–41.9	29.9 ± 7.4	16.8–46.77	<0.05
WC, cm	95.4 ± 15.0	67.5–128.6	94.9 ± 17.6	65.0–131.0	0.862
HC, cm	103.1 ± 10.5	83.0–127.0	111.2 ± 16.3	77.5–153.0	<0.001
FM					
FM, %	23.1 ± 9.3	5.3–41.3	38.8 ± 10.9	11.0–57.9	<0.001
FM, kg	22.3 ± 12.8	3.1–54.7	35.2 ± 17.7	5.9–83.9	<0.001
FMI, kg/m^2	6.8 ± 3.8	1.0–16.7	12.3 ± 6.0	1.9–26.3	<0.001
AT, l					
SAT total	20.6 ± 8.5	7.7–44.3	33.8 ± 15.1	11.0–75.9	<0.001
SAT arms	2.5 ± 0.9	0.3–5.4	3.4 ± 1.5	1.0–7.5	<0.001
SAT trunk	9.6 ± 4.9	2.6–22.1	15.9 ± 7.8	3.3–36.2	<0.001
SAT legs	8.5 ± 3.3	2.9–18.4	15.2 ± 6.7	5.2–34.9	<0.001
VAT	3.9 ± 2.3	0.6–9.3	2.0 ± 1.2	0.3–5.8	<0.001
BrAT	–	–	1.7 ± 0.9	0.4–4.4	
TBW					
TBW, l	51.2 ± 9.0	36.4–76.5	38.9 ± 7.3	25.6–56.8	<0.001
ECW, l	17.8 ± 2.4	13.2–25.7	15.3 ± 2.6	10.5–23.5	<0.001
ICW, l	30.0 ± 3.7	22.1–39.6	23.2 ± 5.5	14.2–37.0	<0.001
SM, kg					
SM total	34.1 ± 5.8	23.3–49.7	22.8 ± 4.2	14.4–34.8	<0.001
SM arms	5.0 ± 0.9	3.2–6.8	3.0 ± 0.6	1.8–4.6	<0.001
SM trunk	12.8 ± 2.5	8.1–19.3	8.1 ± 1.7	4.8–13.5	<0.001
SM legs	16.4 ± 3.3	11.1–24.4	11.7 ± 2.2	6.8–17.5	<0.001
ORGAN AND BONE MASS, kg					
Bone mass	5.4 ± 0.7	4.2–6.8	4.5 ± 0.7	3.0–7.3	<0.001
Brain	1.61 ± 0.12	1.34–1.96	1.45 ± 0.09	1.23–1.75	<0.001
Liver	1.81 ± 0.39	1.16–2.87	1.61 ± 0.32	0.94–2.59	<0.001
Heart	0.34 ± 0.08	0.22–0.57	0.28 ± 0.07	0.17–0.54	<0.001
Kidneys	0.32 ± 0.06	0.20–0.44	0.29 ± 0.08	0.16–0.54	<0.05
Spleen	0.33 ± 0.11	0.17–0.67	0.24 ± 0.10	0.09–0.56	<0.001
Residual	24.0 ± 3.6	16.2–33.7	18.7 ± 3.2	13.0–28.5	<0.001

Comparison between men (n = 58) and women (n = 117) between 20 and 40 years.

**A detailed description of the study protocol, inclusion criteria, subjects, and methods has been published previously (Later et al., 2010). BMI, body mass index; WC, waist circumference; HC, hip circumference; FM, fat mass; FMI, fat mass index; AT, adipose tissue; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; BrAT, breast adipose tissue; TBW, total body water; ECW, extracellular water; ICW, intracellular water; SM, skeletal muscle mass.*

variance was increased. When compared with the other organs, brain relates differently to FFM (i.e., in adults, brain mass does not change much with body weight); therefore, in the last model brain mass was added which reduced the intercept from 410 to non-significant, i.e., 54 kcal/day (Heymsfield et al., 2012).

Normalization of REE for FFM using regression analysis is a statistical rather than a physiological approach (Brozek and Grande, 1955; Garby et al., 1988). FFM is chemically defined as the mass of the body when ether-soluble material has been removed (body weight—FM). However, even though FFM does not differ much from the physiologic lean body mass which is the mass of all tissues in the body excluding adipose tissue (Garrow, 1974), FFM remains a heterogeneous compartment and the specific metabolic rate of FFM decreases with increasing FFM. This is explained by an increase in adipose tissue-derived FFM (water, protein, and minerals) with increasing body weight, but also by a concomitant decrease in the proportion of high metabolically active organ mass (brain, liver, kidney, and heart mass) compared to low metabolically active lean body mass (connective tissue, skeletal muscle mass, bone mass, Wang et al., 2000).

Besides FFM, FM explains a small additional proportion in REE variance, especially in populations with a wide range of adiposity (Nelson et al., 1992). The contribution of FM appears to be around 0.15–0.2 of the contribution of the same mass of FFM

(Garrow and Webster, 1985; Ravussin et al., 1986; Nelson et al., 1992) or even a greater proportion of that of the equivalent FFM in patients with type 2 diabetes (Bitz et al., 2004). Physiological interpretation of the regression coefficient for FM is difficult and can only partly be accomplished by the low metabolic rate of adipose tissue (i.e., 11.3–14.3 kJ × kg lipid^{-1} × 24 h^{-1}, Hallgren et al., 1989; Müller et al., 2009). In mice, the contribution of FM to the variance in REE is substantially greater than predicted from the metabolic cost of adipose tissue *per se* (Kaiyala et al., 2010). The mechanism underlying this effect may be an indirect impact of FM on REE (Bosy-Westphal et al., 2009). In this regard, gains and losses in FM are assumed to trigger compensatory adjustments in REE (e.g., an adipose-tissue specific control of thermogenesis, Dulloo and Jacquet, 1998) and adipose tissue may be coupled to energy homeostasis by "adiposity signals" like leptin and insulin that act on the central nervous system and affect autonomic and behavioral outputs that are directed toward a restoration of energy or fat balance (Morton et al., 2006). These factors might contribute to an increase in REE in obesity and might partly explain how FM can be a major determinant of REE although adipose tissue itself only has a low metabolic rate (Kaiyala et al., 2010).

However, the contribution of FM to metabolic rate in humans largely remains causally enigmatic. The complexity of this contribution is illustrated by our previous results which show that the regression coefficients for FFM and FM differ between different degrees of adiposity (Bosy-Westphal et al., 2009) thus inevitably leading to a yet unsolved problem of REE normalization in different degrees of adiposity when using the same regression equation. Insufficient normalization of energy expenditure also led to the misinterpretation of a low metabolic rate in ob/ob mice (see Butler and Kozak, 2010; Tschöp et al., 2011 for review).

The aim of this contribution is to show that the most established method for normalization of REE based on FFM and FM may lead to spurious conclusions about the phenomenon of weight loss-associated adaptive thermogenesis (i.e., a reduction of REE beyond what is explained by a change in body composition). Adaptive thermogenesis, in obese people who have lost weight, may be overestimated or even seen as an artefact explained by the inadequate normalization for FM. Likewise, we hypothesize that the common procedure of REE normalization for FFM and FM becomes awkward when the metabolic rates of lean and obese persons are compared. In order to test our hypotheses, we compared different methods of REE normalization (1) for FFM and FM, (2) including %FM as a measure of adiposity and (3) based on organ and tissue masses in people with different degrees of adiposity as well as intraindividually before and after weight loss.

METHODS

STUDY PROTOCOLS AND SUBJECTS

Data analysis involved measures of body composition and REE in individuals who took part in previous studies at the Institut für Humanernährung und Lebensmittelkunde, Christian-Albrechts-University Kiel. Briefly, healthy Caucasian men and women were recruited from the general public. All subjects had a normal physical examination, no use of lipid-lowering, hypoglycaemic or antihypertensive medication, no history of cardiovascular or metabolic disease, and a normal thyroid function. Women were non-pregnant and non-lactating. A total number of 301 men and women with a wide age and BMI range (aged 18–78 years with a BMI between 16.8 and 58.7 kg/m^2 = study population 1; **Tables 2** and **3**) were analyzed cross-sectionally including the analysis of organ and tissue masses. In a longitudinal analysis, healthy overweight and obese participants were recruited for a dietary-weight loss intervention trial. In a subsample of 47 men and women organ and tissue masses were analyzed before and after weight loss (study population 2; **Table 4**). Study population 3 (**Table 5**; Bosy-Westphal et al., 2013) consisted of 110 women whose body composition before and after weight loss was assessed by densitometry only. Inclusion criteria and recruitment of participants for the dietary intervention trial was described previously (Bosy-Westphal et al., 2009). The weight loss program consisted of weekly individual counseling by a registered dietician and a 13 ± 3 week low-calorie, nutritionally balanced self-selected diet containing 800–1000 kcal/day where of 434 kcal/day were supplied as a very-low-energy-diet (BCM©-Diät, PreCon, Darmstadt, Germany, ingestion of two shakes/day provided all nutrients according to RDA, 37.3 g protein, 38.8 g carbohydrate, and 13.5 g fat). The studies were approved by the medical faculty ethics committee of the Christian-Albrechts-University Kiel. All subjects provided their fully informed and written consent before participation.

ANTHROPOMETRIC MEASUREMENTS AND BODY COMPOSITION ANALYSIS

Body weight (±100 g) was measured in underwear on an electronic Tanita scale coupled to the BOD-PODTM system. Height was measured without shoes on a stadiometer (seca, Hamburg Germany) to the nearest 0.5 cm.

MAGNETIC RESONANCE IMAGING

Volumes of internal organs were assessed by MRI (Magnetom Avanto 1.5-T, Siemens Medical Systems, Erlangen, Germany). Subjects were examined in a supine position with their arms extended above their heads. Transversal images were obtained

Table 2 | Regression equations for prediction of resting energy expenditure from two different models of body composition analysis (*n* = 301 men and women, BMI 28.4 ± 6.1 kg/m^2, age 40.9 ± 13.9 years, *study population 1*).

REE, MJ/day =	R^2	SEE
0.088 × FFM + 0.027 × FM + 1.237	0.80	0.54
0.788 × liver + 1.004 × brain + 0.064 × SM + 0.057 × RM + 0.022 × AT + 1.891 × kidneys – 0.134	0.84	0.49

SM, skeletal muscle; RM, residual mass; AT, adipose tissue.

Table 3 | Comparison of basal characteristics and resting energy expenditure adjusted for body composition between normal weight, overweight, and obese participants of *study population 1*.

	Normal weight $n = 105$	Over weight $n = 92$	Obese $n = 104$
Age, years	39.6 ± 15.0	46.2 ± 14.4	37.6 ± 10.7
BMI, kg/m^2	22.4 ± 1.9	27.4 ± 1.6	37.6 ± 10.7‡
FM, %	24.2 ± 9.2	30.5 ± 8.7	43.6 ± 8.2‡
FM, kg	15.9 ± 6.1	25.3 ± 6.8	45.3 ± 12.4‡
Skeletal muscle mass, kg	24.7 ± 5.8	27.8 ± 6.5	30.9 ± 7.3‡
Skeletal muscle mass, kg/FFM, kg	0.49 ± 0.07	0.48 ± 0.11	0.53 ± 0.06‡
Organ mass, kg	3.44 ± 0.37	3.86 ± 0.44	4.05 ± 0.49‡
Organ mass, kg/ FFM, kg	0.070 ± 0.008	0.067 ± 0.009	0.071 ± 0.009‡
$REE_{measured}$, MJ/day	6.08 ± 0.88	7.01 ± 1.00	7.69 ± 1.14‡
$REE_{measured-predicted\ according\ to\ organ\ and\ tissue\ masses}$, MJ/day	0.09 ± 0.46	0.09 ± 0.50	0.16 ± 0.61**
$REE_{measured-predicted\ according\ to\ FFM\ and\ FM\ regression\ in\ normal\ weight\ subjects}$[#], MJ/day	−0.04 ± 0.44	0.01 ± 0.54	0.35 ± 0.63***‡
$REE_{measured-predicted\ according\ to\ FFM\ and\ FM\ regression\ in\ obese\ subjects}$[##], MJ/day	−0.33 ± 0.45***	−0.36 ± 0.52***	−0.03 ± 0.62‡

***P < 0.01; ***P < 0.001 difference between measured and predicted REE, paired t-test.*

‡P < 0.05 significantly different from normal and overweight subjects, ANOVA.

[#] Regression equation: REE, MJ/day = 0.084 × FFM, kg + 0.007 × FM, kg + 1.748, $R^2 = 0.75$; SEE = 0.44.

[##] Regression equation: REE, MJ/day = 0.087 × FFM, kg + 0.015 × FM, kg + 1.963, $R^2 = 0.71$; SEE = 0.62.

Table 4 | Comparison of body composition and resting energy expenditure between baseline and after weight loss ($n = 47$ males and females, *study population 2* with organ-tissue analysis).

	Before weight loss	After weight loss
Age, years	36.3 ± 6.3	
Weight, kg	105.5 ± 17.9	95.1 ± 16.6***
BMI, kg/m^2	35.6 ± 4.5	32.1 ± 4.3***
FFM, kg	58.8 ± 11.4	58.2 ± 10.8
FM, kg	46.8 ± 13.7	36.9 ± 14.5***
Skeletal muscle, kg	32.0 ± 8.1	27.5 ± 6.7***
Organ mass, kg	4.03 ± 0.50	3.84 ± 0.48***
REE, MJ/day	7.73 ± 1.24	7.13 ± 0.96***
$REE_{measured-predicted\ according\ to\ FFM\ and\ FM\ regression\ before\ weight\ loss}$, MJ/day	0.03 ± 0.70	−0.32 ± 0.72***
$REE_{measured-predicted\ according\ to\ organ\ and\ tissue\ masses}$, MJ/day	0.19 ± 0.66	0.16 ± 0.55

****P < 0.001 difference between before and after weight loss, paired t-test.*

Organ mass = sum of brain, heart, liver, and kidney masses.

from wrist to ankle by using a contiguous axial T1 weighted gradient-echo sequence (TR 157 ms, TE 4 ms, flip angle 70°, voxel size 3.9 × 2 × 8 mm^3). Only images from the head, abdominal and thoracic regions were included in the present analysis. The protocol for the brain comprised contiguous 4 mm slices with 1 mm inter-slice gaps (TR 313 ms, TE 14 ms). For the rest of the body, images were obtained with 8 mm slice thickness and 2 mm inter-slice gaps. Image acquisition for volumetric assessment of the thoracic and abdominal region was obtained in breath-hold and heart mass was assessed using a breath navigated and pulse triggered T2-weighted HASTE sequence, (imaging parameters: TR 700 ms, TE 24 ms, flip angle 160°, voxel size 2.2 × 1.3 × 8 mm, turbo factor 106). All images were segmented manually (Slice-O-Matic, Tomovision 4.3 Software, Montreal, Canada). Total organ volume was determined from the sum of all areas (cm^2) multiplied by slice thickness. Volume data were transformed into organ mass using the following densities: 1.036 g/cm^3 for brain, 1.06 g/cm^3 for heart and liver and 1.05 g/cm^3 for kidneys (Elia, 1992).

DENSITOMETRY

Air-displacement plethysmography was performed using the BOD-PODTM device (Life Measurement Instruments., Concord, CA, USA). Subjects were measured in tight fitting underwear and a swimming cap. Two repeated measurements of body volume were performed and averaged. Measured thoracic lung volume was subtracted from body volume. BOD-POD® software was used to calculate body density as body weight divided by body volume and FM% using Siri's equation (Siri, 1993). Fat free mass (FFM, kg) was calculated as: weight (kg)—FM (kg). The coefficient of variation for repeated measurements of %FM was 2.4%.

RESTING ENERGY EXPENDITURE

Indirect calorimetry was performed in the morning between 7.30 and 9.00 a.m. after an overnight fast (ventilated hood system: Vmax Spectra 29 n; SensorMedics BV, Bilthoven, Netherlands; software Vmax, version 12-1A). The minimum duration of measurement was 35 min and the first 10 min were discarded. Flow calibration was performed by a 3L-syringe and gas analyzers were calibrated before and every 5 min during the run. Data were collected every 20 s and acquired VO2 and VCO2 were converted to REE (kcal/24 h) using the abbreviated equation of Weir. The CVs for repeated REE-measurements were 5.2%.

REE was normalized for FFM and FM by regression analysis (REEadjusted FFM + FM) and also accounting for detailed body composition by subtracting REE calculated from organ and tissue masses (REEc) from measured REE (REEmeasured-calculated). Calculation of REE was based on the sum of eight body compartments (brain, heart, liver, kidneys, skeletal muscle mass, bone mass, adipose tissue, and residual mass) times the corresponding tissue-respiration rate, using the specific tissue-metabolic rates as reported by Elia (1992; see Müller et al., 2002). Residual mass was calculated as body mass minus the sum of brain, heart, liver, kidneys, skeletal muscle mass, and adipose tissue. The metabolic activity of residual mass was assumed to be 30 kJ/kg/day (Bosy-Westphal et al., 2009).

REEc (kJ/day) = (1008 × brain mass) + (840 × liver mass) + (1848 × heart mass) + (1848 × kidney mass) + (55 × skeletal muscle mass) + (19 × adipose tissue) + (30 × residual mass).

Adipose tissue was calculated from FM assuming a fat content of 90%.

Skeletal muscle mass was derived from appendicular lean soft tissue measured by DXA (Hologic Discovery A densitometer, Hologic, Inc., Bedford, Massachusetts, USA) using equations validated against whole body MRI (Kim et al., 2002).

STATISTICS

Data are expressed as means ±SD. Comparisons between independent groups were analyzed by ANOVA using Bonferroni *post-hoc* test for comparisons between three BMI groups. Intraindividual comparisons between baseline values and after weight loss were analyzed using paired samples *t*-test. Relationships between variables were sought by correlation analysis (Pearson's r). Two-tailed *P*-values <0.05 were considered to indicate statistical significance. Data analyses were performed with SPSS statistical software (SPSS 15.0, Inc., Chicago, USA).

RESULTS

IMPACT OF DIFFERENT WAYS TO NORMALIZE REE IN NORMAL AND OVERWEIGHT SUBJECTS

The results of stepwise regression analyses predicting REE from two different models of body composition analysis are given in **Table 2** for *study population 1*. The coefficient of determination is only marginally lower and standard error of estimate is slightly higher for the prediction model based on absolute values of FFM and FM when compared with a prediction model based on organ and tissue masses. Both models were not significantly improved by the inclusion of gender, age, or %FM.

Comparing the different models for adjusting REE between normal weight, overweight and obese participants reveals that REE predicted from organ and tissue masses does not fully explain the higher REE in obese subjects whereas the difference between measured and predicted REE was not significant in normal and overweight subjects (**Table 3**). However, there was no significant difference in $REE_{measured-calculated\ by\ organ\ and\ tissue\ masses}$ when comparing obese vs. lean/overweight participants. By contrast, the difference in REE between normal-/overweight and obese participants was significant after adjusting REE using regression analysis based on FFM and FM. Transferring the regression equation derived in obese participants to normal-/overweight subjects' leads to a seemingly lowered metabolic rate in these groups. Conversely, an equation derived in normal weight participants and applied to normalize REE in obesity leads to the result of an elevated metabolic rate in obese subjects.

Table 4 shows the results for REE adjusted for body composition before and after weight loss in *study population 2* (with detailed analysis of organ and tissue masses). Mean weight loss was -10.4 ± 4.2 kg ($p < 0.001$) and mainly consisted of FM. Lean mass was preserved and did not significantly differ between baseline and after weight loss. REE adjusted for FFM and FM was based on a regression equation developed at baseline and showed a significant drop in metabolic rate after weight loss ($p < 0.001$) whereas REE adjusted for changes in organ and tissue masses did not change with follow-up.

In *study population 3* (see **Table 5**), body composition analysis was performed by a 2-compartment model only. Similar to *study population 2*, weight loss mainly consisted of FM and adjusting REE for FFM and FM based on regression analysis performed at baseline led to a lower adjusted REE after weight loss. In this group of women we also compared REE measured by indirect calorimetry to REE predicted from %FM-specific REE-prediction equations (based on FFM and FM) that were derived in a large female database and have been previously published by our group (Bosy-Westphal et al., 2009). The comparison between adjusted REEs before and after weight loss showed no significant differences.

Using data from *study population 1*, we analyzed the contribution of different organ masses to FFM with increasing adiposity (**Table 6**). Surprisingly, the contribution of liver, heart (women only), kidney, and skeletal muscle masses per kg FFM increased with increasing %FM in both genders but the ratios of brain/FFM (women only) and residual mass/FFM that showed an inverse association with %FM. Accordingly, regression analysis using $REE_{calculated\ from\ organ\ and\ tissue\ masses}$ (MJ)/FFM (kg) as the dependent variable and %FM as the independent variable revealed a significant positive relationship ($REE_{calculated\ from\ organ\ and\ tissue\ masses}$ /FFM = 0.001 × %FM + 0.105; $R^2 = 0.44$).

DISCUSSION

REE NORMALIZATION IN LEAN AND OBESE PEOPLE AND BEFORE AND AFTER WEIGHT LOSS

Our results show that an "REE vs. FFM and FM equation" that (1) was derived in obese participants and applied to lean people or (2) was derived before weight loss and applied to data after weight

Table 5 | Comparison of body composition and resting energy expenditure between baseline and after weight loss (*n* = 110 females, *study population 3* without organ-tissue analysis).

	Before weight loss	After weight loss
Age, years	36.2 ± 9.7	
Weight, kg	107.9 ± 21.8	93.9 ± 17.1***
BMI, kg/m^2	38.0 ± 7.0	33.0 ± 4.9***
FFM, kg	54.3 ± 7.4	52.8 ± 6.1**
FM, kg	53.6 ± 16.6	41.1 ± 13.3***
FM, %	48.8 ± 6.3	42.8 ± 7.1***
REE, MJ/day	7.46 ± 1.06	6.79 ± 0.84***
$REE_{measured-predicted\ according\ to\ FFM\ and\ FM\ regression\ before\ weight\ loss}$, MJ/day	0.01 ± 0.64	−0.23 ± 0.51***
$REE_{measured-predicted\ according\ to\ \%FM\text{-}specific\ FFM\text{-}regressions}$, MJ/day†	−1.23 ± 0.65	−1.28 ± 0.58

***P < 0.01; ***P < 0.001 difference between before and after weight loss, paired t-test.*

†Equations were published in Bosy-Westphal et al. (2009).

Table 6 | Coefficients of correlation between organ and tissue masses/FFM and adiposity (%FM) stratified by gender in *study population 1*.

	%FM vs.	
	Women (*n* = 179)	Men (*n* = 122)
Skeletal muscle (kg)/FFM (kg)	0.30***	0.27**
Residual mass (kg)/FFM (kg)	−0.57***	−0.44***
Brain mass (kg)/FFM (kg)	−0.25**	−0.05
Liver mass (kg)/FFM (kg)	0.39***	0.53***
Heart mass (kg)/FFM (kg)	0.17*	−0.18
Kidney masses (kg)/FFM (kg)	0.38***	0.21*

**P < 0.05; **P < 0.01; ***P < 0.001.*

loss leads to the erroneous conclusion of a lower metabolic rate (i) in lean persons (**Table 3**) and (ii) after weight loss (**Table 4**). By contrast, the normalization of REE for organ and tissue masses (REEmeasured—REE calculated from organ and tissue masses) was not significantly different between lean and obese or between baseline and after weight loss.

However, the measurement of organ and tissue masses to normalize REE is not suitable in daily clinical practice and has only been used in a limited number of studies. Only recently, a substantial drop in specific REE (−5.4 ± 1.1 kcal/day) has been deduced after diet and exercise induced massive weight loss (−57.6 kg) despite a relative preservation of FFM (Johannsen et al., 2012). This diagnosis was based on a regression analysis that predicted REE from baseline levels of FFM, FM, age, and sex and was applied to FFM and FM measured after weight loss. The authors concluded that the high difference between measured and predicted REE after weight loss corresponds to a dramatic metabolic slowing that cannot be explained by changes in body composition. However, it is possible that in this study "metabolic slowing" did not occur *despite* the preservation of lean mass but as a direct consequence of it. The preferential loss in FM (−47.1 kg, 83 ± 8% of weight loss) likely contributed to an overestimation of adaptive thermogenesis because the regression analysis from baseline values was valid for a mean %FM of 49 ± 5%. After weight loss, participants had a mean %FM of 28 ± 10% only. In a previous publication we have shown that the regression coefficients for FFM and FM differ between different degrees of adiposity (Bosy-Westphal et al., 2009). Therefore, a regression analysis derived from participants with a higher %FM cannot be used to normalize REE in a leaner group of people without bias. In line with this argumentation, the application of our previously published %FM-specific regression equations (Bosy-Westphal et al., 2009) to normalize REE before and after weight loss in the article of Johannsen et al. (2012) leads to a comparable accuracy of REE prediction at both time points (REEmeasured—predicted at baseline + 120 kcal/day and after weight loss + 112 kcal/day). The small inaccuracy of REE prediction at both time points is likely due to differences in body composition analysis (DXA vs. BIA) and the fact that our equations apply to women only. Using these equations to normalize REE also eliminated the difference in REE before and after weight loss (**Table 5**).

A physiologic explanation of the seemingly higher metabolic rate before weight loss when adipocytes are large and filled with lipids compared to the smaller fat cells after weight loss induced lipolysis remains unclear. It is tempting to speculate that obesity associated co-morbidities (Bosy-Westphal et al., 2008) as well as the endocrine function of adipose tissue (Kaiyala and Schwartz, 2011) contribute to an elevation of REE that is normalized after weight loss. However, the fact that REE normalized for organ and tissue masses can be applied to lean and obese people with sufficient accuracy (**Table 3**, Bosy-Westphal et al., 2004; Wang et al., 2012) indicates that changes in the regression coefficients of FFM and FM with increasing adiposity may be partly explained by changes in the composition of lean mass (i.e., the ratio of high to low metabolic organs). In line with this observation, metabolic activity of FFM (e.g., REE predicted from organ and tissue masses/FFM) increased with increasing %FM. This may be due to increased masses of liver, heart and kidneys per kg FFM with increasing %FM whereas brain mass per FFM and low metabolically active residual mass/FFM decreased (**Table 6**).

NORMALIZATION OF REE BASED ON ORGAN AND TISSUE MASSES

The concept of relating metabolic rate to organ size was introduced by Holliday et al. as early as in 1967. Brain, heart, liver,

and kidneys comprise only 5–6% of body weight, but contribute to >80% of REE, whereas other components such as muscle, adipose tissue or bone mass have low specific resting metabolic rates (Smith and Hoyer, 1962; Grande, 1980; Elia, 1992). However, initial studies published between 1992 and 1997 did not find a significant contribution of tissue masses to the REE variance beyond that explained in FFM (Deriaz et al., 1992; McNeill et al., 1995; Sparti et al., 1997). This may be due to the small numbers of subjects investigated, low inter-individual differences in body composition, and methodological limitations that did not allow a differentiated volumetric assessment of all organs and tissues.

Today, functional body composition analysis at the organ and tissue level adds to our understanding of inter-individual variance in REE (Gallagher et al., 2000, 2006; Illner et al., 2000; Hsu et al., 2003; Midorikawa et al., 2007; for a review see Müller et al., 2002, 2009). Differences in organ and tissue mass contribute to differences in REE between underweight, overweight and obese subjects (cf. Bosy-Westphal et al., 2004), Sumo wrestlers and untrained college students (Midorikawa et al., 2007) as well as between African American and white adults (Gallagher et al., 2006). Organ and tissue modeling of REE has also shown that the specific metabolic rates apply equally well in both genders (i.e., differences in REE normalized for FFM between men and women are explained by sex-differences in FFM-composition, Wang et al., 2011). A lower mass of high metabolic rate organs also contributes to the lower REE in elderly individuals (Bosy-Westphal et al., 2003) but did not fully account for the differences in REE observed between young and elderly people (Gallagher et al., 2000). This is also confirmed in a recent analysis performed in a greater study population (comprising 131 adults aged 21–73 years with a BMI $< 30\,kg/m^2$) which showed that the specific metabolic rates of major organs (brain, heart, liver, and kidneys) published by Elia (1992) are valid in the younger age groups but were estimated to be 3% lower in the group >50 years (Wang et al., 2010).

In addition, a greater mass of high metabolic rate organs does not fully explain the higher metabolic rate observed in children (Hsu et al., 2003). This is likely explained by the metabolic costs of growth (Holliday, 1971).

Inter-species comparison shows that specific organ metabolic rate varies with body mass, with higher energy expenditure per unit organ mass in smaller mammals (Couture and Hulbert, 1995). There is, however, no evidence for a mass dependency of specific organ metabolic rate in humans with a range of body mass from 44 to 104 kg and a normal FM (Later et al., 2008).

PROPOSAL OF A NEW WAY OF REE NORMALIZATION INCLUDING INFORMATION ON %FM

REE normalization based on organ and tissue masses is expensive, time-consuming and methodically complex that is not without limitation (e.g., assumption about the lipid content of adipose tissue and liver) and confined to smaller sample sizes. Normalization of REE based on FFM and FM therefore remains indispensable in daily practice and the majority of scientific studies. We propose the use of %FM-specific regression equations for REE vs. FFM and FM (Bosy-Westphal et al., 2009). The advantage of this method is a plausible approach that provides new insights on the contribution of adiposity to metabolic rate. The information on adiposity is an advantage over the commonly applied normalization of REE using FFM and FM in kg only. FM in kg does not reflect adiposity of the body because a large and heavy person and a smaller person can have the same amount of FM but differ greatly in their adiposity (%FM). Thus, the influence of adiposity on the composition of lean mass (**Table 6**) or the impact of obesity on co-morbidities and the endocrine function of adipose tissue can only be taken into account by REE normalization that includes information on adiposity. This argument is supported by our finding, that %FM-specific regression of REE vs. FFM and FM (derived from Bosy-Westphal et al., 2009) revealed no difference in metabolic rate before and after weight loss whereas the conventional approach using only absolute values of FFM and FM for normalization leads to a significant difference between measured and predicted REE.

STUDY LIMITATIONS

FFM was measured by air-displacement plethysmography (densitometry, **Table 3**) that is known to overestimate the loss in FM (and underestimate the loss in lean mass) during weight loss due to similar densities of fat and water. This bias may have contributed to an overestimation of lean mass after weight loss that could explain the significantly lower REE adjusted for FFM and FM. In addition, some authors found that the hydration of FFM did not normalize after weight loss (Das et al., 2003). The higher hydration of FFM in obese and weight reduced subjects may thus contribute to an overestimation of FFM. Therefore, the lack of all two compartment methods to accurately assess changes in body composition with weight loss may mimic a reduction in specific metabolic rate.

The finding of a seemingly higher metabolic activity of FFM with increasing adiposity (due to an increase in organ mass/FFM) was unexpected and may have been overestimated by an increased liver fat content with increasing adiposity. Future studies should investigate the contribution of liver fat to the specific metabolic rate of this tissue.

In contrast to the present findings, in a previous publication conducted in overweight and obese women who lost weight in response to a low-calorie diet for 3 months we found that about 40% of the decline in REE were not explained by a decrease in organ and tissue masses and were thus attributed to adaptive thermogenesis (Bosy-Westphal et al., 2009). The reason for the discrepant findings remains unclear. However, the population of the present manuscript differs from our former publication and also included men. Organ mass and skeletal muscle mass were both higher in the present population. In addition, the loss in skeletal muscle mass was also higher. Because appendicular lean soft tissue measured by DXA was used to calculate skeletal muscle mass, and these equations differ for men and women, the relative maintenance of muscle mass in the former publication could have been overestimated thus leading to a reduction in REE adjusted for organ and tissue masses. These methodological limitations in addition to uncertainties about organ lipid content and their impact on organ specific metabolic rate as well as organ hydration in response to weight loss add to uncertainty about the 'quantification' of adaptive thermogenesis in human weight regulation,

which is therefore likely to remain more of a concept than a measurable entity (Dulloo et al., 2012; Müller and Bosy-Westphal, 2012).

Finally, our %FM-specific REE normalization approach (Bosy-Westphal et al., 2009) comprises only women because a large number of individuals in the same %FM-category differing widely in FFM is required to derive %FM-specific equations predicting REE from FFM. However, having five different equations for REE-normalization depending on %FM has some drawbacks due to abrupt changes depending on the cut-off. Future studies should develop a more continuous way to adjust REE for FFM and FM.

The prediction of skeletal muscle mass from lean soft tissue of the extremities measured by DXA cannot account for the higher content of connective tissue with increasing adiposity (Schautz et al., 2012) and may therefore overestimate skeletal muscle mass in obesity. In addition, the assumption of a constant density (i.e., lipid content) of adipose tissue and liver mass is likely violated in obesity and contributes to a bias when normalizing REE for organ and tissue masses in obese patients.

The impact of adiposity associated co-morbidities (SNS-activity, insulin resistance, blood pressure) or endocrine function (fT3, leptin etc.) was analyzed in our previous publications (Bosy-Westphal et al., 2008) and is beyond the scope of the present paper.

In summary, the normalization of REE for body composition is not trivial when comparing people with a wide range of adiposity as well as before and after substantial weight loss. The most established method for normalization of REE based on FFM and FM may lead to spurious conclusions about metabolic rate in obesity and the phenomenon of weight loss-associated adaptive thermogenesis. Organ-tissue based models are superior to equations based on FFM and FM. However, information on organ and tissue mass is rarely available and using %FM in addition to FFM and FM for adjusting REE may account for the increase in specific metabolic rate of lean mass with increasing adiposity and thus provide new insights into the old controversy about the impact of specific REE on the cause and consequence of obesity.

FUNDING

The study was funded by a grant of the German Ministry of Education and Research (BMBF 0315681), BMBF Competent Network of Obesity (CNO) and the German Research Foundation (DFG Bo 3296/1-1 and DFG Mü 714/ 8-3).

REFERENCES

Arch, J. R., Hislop, D., Wang, S. J., and Speakman, J. R. (2006). Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals. *Int. J. Obes. (Lond.)* 30, 1322–1331.

Bitz, C., Toubro, S., Larsen, T. M., Harder, H., Rennie, K. L., Jebb, S. A., et al. (2004). Increased 24-h energy expenditure in type 2 diabetes. *Diabetes Care* 27, 2416–2421.

Bosy-Westphal, A., Eichhorn, C., Kutzner, D., Illner, K., Heller, M., and Müller, M. J. (2003). Age-related decline in resting energy expenditure is explained by alterations in metabolically active components of fat free mass. *J. Nutr.* 133, 2356–2362.

Bosy-Westphal, A., Goele, K., Later, W., Hitze, B., Kossel, E., Settler, U., et al. (2009). Contribution of individual organ mass loss to weight loss-associated decline in resting energy expenditure. *Am. J. Clin. Nutr.* 90, 993–1001.

Bosy-Westphal, A., Müller, M. J., Boschmann, M., Klaus, S., Kreymann, G., Lührmann, P. M., et al. (2009). Grade of adiposity affects the impact of fat mass on resting energy expenditure in women. *Br. J. Nutr.* 101, 474–477.

Bosy-Westphal, A., Reinecke, U., Schlörke, T., Illner, K., Kutzner, D., Heller, M., et al. (2004). Effect of organ and tissue masses on resting energy expenditure in underweight, normal weight and obese adults. *Int. J. Obes. Relat. Metab. Disord.* 28, 72–79.

Bosy-Westphal, A., Schautz, B., Lagerpusch, M., Pourhassan, M., Braun, W., Goele, K., et al. (2013). Effect of weight loss and regain on adipose tissue distribution, composition of lean mass, and resting energy expenditure in young overweight and obese adults. *Int. J. Obes. (Lond.)* doi: 10.1038/ijo.2013.1. [Epub ahead of print].

Bosy-Westphal, A., Wolf, A., Bührens, F., Hitze, B., Czech, N., Mönig, H., et al. (2008). Familial influences and obesity-associated metabolic risk factors contribute to the variation in resting energy expenditure: the Kiel Obesity Prevention Study. *Am. J. Clin. Nutr.* 87, 1695–1701.

Brozek, J., and Grande, F. (1955). Body composition and basal metabolism in man: correlation analysis vs. physiologic approach. *Hum. Biol.* 27, 22–31.

Butler, A. A., and Kozak, L. P. (2010). A recurring problem with the analysis of energy expenditure in genetic models expressing lean and obese phenotypes. *Diabetes* 59, 323–329.

Couture, P., and Hulbert, A. J. (1995). Relationship between body mass, tissue metabolic rate, and sodium pump activity in mammalian liver and kidney. *Am. J. Physiol.* 268, R641–R650.

Das, S. K., Roberts, S. B., Kehayias, J. J., Wang, J., Hsu, L. K., Shikora, S. A., et al. (2003). Body composition assessment in extreme obesity and after massive weight loss induced by gastric bypass surgery. *Am. J. Phys. Endocrinol. Metab.* 284, E1080–E1088.

Deriaz, O., Fournier, G., Tremblay, A., Despres, J.-P., and Bouchard, C. (1992). Lean-body mass composition and resting energy expenditure before and after long-term overfeeding. *Am. J. Clin. Nutr.* 56, 840–847.

Dulloo, A. G., and Jacquet, J. (1998). Adaptive reduction in basal metabolic rate in response to food deprivation in humans: a role for feedback signals from fat stores. *Am. J. Clin. Nutr.* 68, 599–606.

Dulloo, A. G., Jacquet, J., Montani, J. P., and Schutz, Y. (2012). Adaptive thermogenesis in human body weight regulation: more of a concept than a measurable entity? *Obes. Rev.* 13(Suppl. 2),105–121.

Elia, M. (1992). "Organ and tissue contribution to metabolic rate," in *Energy Metabolism: Tissue Determinants and Cellular Corollaries,* eds J. M. Kinney and H. N. Tucker (New York, NY: Raven), 61–79.

Gallagher, D., Albu, J., He, Q., Heshka, S., Boxt, L., Krasnow, N., et al. (2006). Small organs with a high metabolic rate explain lower resting energy expenditure in African American than in white adults. *Am. J. Clin. Nutr.* 83, 1062–1067.

Gallagher, D., Allen, A., Wang, Z., Heymsfield, S. B., Krasnow, N. (2000). Smaller organ tissue mass in the elderly fails to explain lower resting metabolic rate. *Ann. N.Y. Acad. Sci.* 904, 449–455.

Garby, L., Garrow, J. S., Jorgensen, B., Lammert, O., Madsen, K., Sorensen, P., et al. (1988). Relation between energy expenditure and body composition in man: specific energy expenditure *in vivo* of fat and fat-free mass. *Eur. J. Clin. Nutr.* 42, 301–305.

Garrow, J. S. (1974). *Energy Balance and Obesity in Man.* New York, NY: American Elsevier Publishing Company Inc.

Garrow, J. S., and Webster, J. (1985). Are pre-obese people energy thrifty? *Lancet* 1, 670–671.

Grande, F. (1980). "Energy expenditure of organs and tissues," in *Assessment of Energy Metabolism in Health and Disease. Report of the First Ross Conference on Medical Research,* ed J. Kinney (Columbus, OH: Ross Laboratories), 88–92.

Hallgren, P., Sjostrom, L., Hedlund, H., Lundell, L., and Olbe, L. (1989). Influence of age, fat cell weight, and obesity on O_2 consumption of human adipose tissue. *Am. J. Physiol.* 256, E467–E474.

Heymsfield, S. B., Thomas, D., Bosy-Westphal, A., Shen, W., Peterson, C. M., and Müller, M. J. (2012). Evolving concepts on adjusting human resting energy expenditure measurements for body size. *Obes. Rev.* 13, 1001–1014.

Holliday, M. A. (1971). Metabolic rate and organ size during growth from infancy to maturity and during late gestation and early infancy. *Pediatrics* 47, 169–179.

Holliday, M. A., Potter, D., Jarrah, A., and Bearg, S. (1967). The relation of metabolic rate to body weight and organ size. *Pediatr. Res.* 1, 185–195.

Hsu, A., Heshka, S., Janumala, I., Song, M. Y., Horlick, M., Krasnow, N., et al. (2003). Larger mass of high-metabolic-rate organs does not explain higher resting energy expenditure in children. *Am. J. Clin. Nutr.* 77, 1506–1511.

Illner, K., Brinkmann, G., Heller, M., Bosy-Westphal, A., and Müller, M. J. (2000). Metabolically active components of fat free mass and resting energy expenditure in nonobese adults. *Am. J. Physiol. Endocrinol. Metab.* 278, E308–E315.

Johannsen, D. L., Knuth, N. D., Huizenga, R., Rood, J. C., Ravussin, E., and Hall, K. D. (2012). Metabolic slowing with massive weight loss despite preservation of fat-free mass. *J. Clin. Endocrinol. Metab.* 97, 2489–2496.

Kaiyala, K. J., Morton, G. J., Leroux, B. G., Ogimoto, K., Wisse, B., and Schwartz, M. W. (2010). Identification of body mass as a major determinant of metabolic rate in mice. *Diabetes* 59, 1657–1666.

Kaiyala, K. J., and Schwartz, M. W. (2011). Toward a more complete and less controversial understanding of energy expenditure and its role in obesity pathogenesis. *Diabetes* 60, 17–23.

Kim, J., Wang, Z., Heymsfield, S. B., Baumgartner, R. N., and Gallagher, D. (2002). Total-body skeletal muscle mass: estimation by a new dual-energy X-ray absorptiometry method. *Am. J. Clin. Nutr.* 76, 378–383.

Korth, O., Bosy-Westphal, A., Zschoche, P., Glüer, C. C., Heller, M., and Müller, M. J. (2007). Influence of methods used in body composition analysis on the prediction of resting energy expenditure. *Eur. J. Clin. Nutr.* 61, 582–589.

Later, W., Bosy-Westphal, A., Hitze, B., Kossel, E., Glüer, C. C., Heller, M., et al. (2008). No evidence of mass dependency of specific organ metabolic rate in healthy humans. *Am. J. Clin. Nutr.* 88, 1004–1009.

Later, W., Bosy-Westphal, A., Kossel, E., Glüer, C. C., Heller, M., and Müller, M. J. (2010). Is the 1975 Reference Man still a suitable reference? *Eur. J. Clin. Nutr.* 64, 1035–1042.

McNeill, G., Foster, M. A., Love, J., and Antfang, V. (1995). Liver and kidney volume and their relationship to metabolic rate at rest. *Proc. Nutr. Soc.* 54, 151A.

Midorikawa, T., Kondo, M., Beekley, M. D., Koizumi, K., and Abe, T. (2007). High REE in Sumo wrestlers attributed to large organ-tissue mass. *Med. Sci. Sports Exerc.* 39, 688–693.

Morton, G. J., Cummings, D. E., Baskin, D. G., Barsh, G. S., and Schwarz, M. W. (2006). Central nervous system control of food intake and body weight. *Nature* 443, 289–295.

Müller, M. J., and Bosy-Westphal, A. (2012). Adaptive thermogenesis with weight loss in humans. *Obesity (Silver Spring).* doi: 10.1002/oby.20027. [Epub ahead of print].

Müller, M. J., Bosy-Westphal, A., Kutzner, D., and Heller, M. (2002). Metabolically active components of fat-free mass and resting energy expenditurein humans: recent lessons from imaging technologies. *Obes. Rev.* 3, 113–122.

Müller, M. J., Bosy-Westphal, A., Later, W., Haas, V., and Heller, M. (2009). Functional body composition: insights into the regulation of energy metabolism and some clinical applications. *Eur. J. Clin. Nutr.* 63, 1045–1056.

Müller, M. J., Langemann, D., Gehrke, I., Later, W., Heller, M., Glüer, C. C., et al. (2011). Effect of constitution on mass of individual organs and their association with metabolic rate in humans–a detailed view on allometric scaling. *PLoS ONE* 6:e22732. doi: 10.1371/journal.pone.0022732

Nelson, K. M., Weinsier, R. L., Long, C. L., and Schutz, Y. (1992). Prediction of resting energy expenditure from fat-free mass and fat mass. *Am. J. Clin. Nutr.* 56, 848–856.

Ravussin, E., Lillioja, S., Anderson, T. E., Christin, L., and Bogardus, C. (1986). Determinants of 24-h energy expenditure in man. Methods and results using a respiratory chamber. *J. Clin. Invest.* 78, 1568–1578.

Schautz, B., Later, W., Heller, M., Müller, M. J., and Bosy-Westphal, A. (2012). Total and regional relationship between lean and fat mass with increasing adiposity-impact for the diagnosis of sarcopenic obesity. *Eur. J. Clin. Nutr.* 66, 1356–1361.

Smith, R. E., and Hoyer, D. J. (1962). Metabolism and cellular function in cold acclimation. *Physiol. Rev.* 42, 60.

Siri, W. E. (1993). Body composition from fluid spaces and density: analysis of methods. 1961. *Nutrition* 9, 480–491. discussion: 480, 492.

Sparti, A., DeLany, J. P., De La Bretonne, J., Sander, G. E., and Bray, G. A. (1997). Relationship between resting metabolic rate and the composition of the fat-free mass. *Metab. Clin. Exp.* 46, 1225–1230.

Tschöp, M. H., Speakman, J. R., Arch, J. R., Auwerx, J., Brüning, J. C., Chan, L., et al. (2011). A guide to analysis of mouse energy metabolism. *Nat. Methods* 9, 57–63.

Wang, Z., Heshka, S., Gallagher, D., Boozer, C. N., Kotler, D. P., and Heymsfield, S. B. (2000). Resting energy expenditure-fat-free mass relationship: new insights provided by body composition modeling. *Am. J. Physiol. Endocrinol. Metab.* 279, E539–E545.

Wang, Z., Ying, Z., Bosy-Westphal, A., Zhang, J., Heller, M., Later, W., et al. (2011). Evaluation of specific metabolic rates of major organs and tissues: comparison between men and women. *Am. J. Hum. Biol.* 23, 333–338.

Wang, Z., Ying, Z., Bosy-Westphal, A., Zhang, J., Heller, M., Later, W., et al. (2012). Evaluation of specific metabolic rates of major organs and tissues: comparison between nonobese and obese women. *Obesity (Silver Spring)* 20, 95–100.

Wang, Z., Ying, Z., Bosy-Westphal, A., Zhang, J., Schautz, B., Later, W., et al. (2010). Specific metabolic rates of major organs and tissues across adulthood: evaluation by mechanistic model of resting energy expenditure. *Am. J. Clin. Nutr.* 92, 1369–1377.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 November 2012; accepted: 28 February 2013; published online: 25 March 2013.

*Citation: Bosy-Westphal A, Braun W, Schautz B and Müller MJ (2013) Issues in characterizing resting energy expenditure in obesity and after weight loss. Front. Physiol. **4**:47. doi: 10.3389/fphys.2013.00047*

This article was submitted to Frontiers in Integrative Physiology, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 01 May 2013
doi: 10.3389/fphys.2013.00094

Practical aspects of estimating energy components in rodents

Jan B. van Klinken[1]*, Sjoerd A. A. van den Berg[2] and Ko Willems van Dijk[1,3]

[1] *Department of Human Genetics, Leiden University Medical Center, Leiden, Netherlands*
[2] *Laboratory for Clinical Chemistry and Hematology, Amphia Hospital, Breda, Netherlands*
[3] *Department of General Internal Medicine, Leiden University Medical Center, Leiden, Netherlands*

Edited by:
Patrick C. Even, AgroParisTech, France

Reviewed by:
Etienne Labussière, INRA, France
Samuel Virtue, University of Cambridge, UK

****Correspondence:***
Jan B. van Klinken, Department of Human Genetics, Leiden University Medical Center, PO Box 9600, Leiden 2300 RC, Netherlands.
e-mail: j.b.van_klinken@lumc.nl

Recently there has been an increasing interest in exploiting computational and statistical techniques for the purpose of component analysis of indirect calorimetry data. Using these methods it becomes possible to dissect daily energy expenditure into its components and to assess the dynamic response of the resting metabolic rate (RMR) to nutritional and pharmacological manipulations. To perform robust component analysis, however, is not straightforward and typically requires the tuning of parameters and the preprocessing of data. Moreover the degree of accuracy that can be attained by these methods depends on the configuration of the system, which must be properly taken into account when setting up experimental studies. Here, we review the methods of Kalman filtering, linear, and penalized spline regression, and minimal energy expenditure estimation in the context of component analysis and discuss their results on high resolution datasets from mice and rats. In addition, we investigate the effect of the sample time, the accuracy of the activity sensor, and the washout time of the chamber on the estimation accuracy. We found that on the high resolution data there was a strong correlation between the results of Kalman filtering and penalized spline (P-spline) regression, except for the activity respiratory quotient (RQ). For low resolution data the basal metabolic rate (BMR) and resting RQ could still be estimated accurately with P-spline regression, having a strong correlation with the high resolution estimate ($R^2 > 0.997$; sample time of 9 min). In contrast, the thermic effect of food (TEF) and activity related energy expenditure (AEE) were more sensitive to a reduction in the sample rate ($R^2 > 0.97$). In conclusion, for component analysis on data generated by single channel systems with continuous data acquisition both Kalman filtering and P-spline regression can be used, while for low resolution data from multichannel systems P-spline regression gives more robust results.

Keywords: indirect calorimetry, rodent models, component analysis, resting metabolic rate, activity related energy expenditure

INTRODUCTION

In the last two decades, metabolic chambers employing open flow indirect calorimetry have become a standard tool in the study of obesity in humans and rodent models. The time-dependent character of the data generated by such devices contains a wealth of information and provides detailed insights into energy metabolism and changes therein due to physical activity (PA), feeding, and experimental interventions. Using the proper experimental protocol and device settings, it becomes possible to quantify the components that make up total energy expenditure (TEE) and to determine the time response to metabolic challenges (Even and Nadkarni, 2012).

Central to component analysis of indirect calorimetry data that is obtained in freely moving animals or humans is the separation of activity and resting energy expenditure. Several mathematical methods have been proposed for this purpose over the years, amongst which linear regression (Ravussin et al., 1986; Kumahara et al., 2004; Bjursell et al., 2008), Kalman filtering (Even et al., 1991), and penalized spline regression (Van Klinken et al., 2012). The correct application of these methods in the analysis of indirect calorimetry data is challenging and care has to be taken that the derived biological parameters provide an accurate reflection of energy metabolism. Moreover, the performance of these methods depends on several factors such as the sampling frequency of the respiratory exchange, the chamber washout time, and the type of activity sensor. Therefore, understanding of how these mathematical methods work and of how experimental settings affect the data and, in turn, the precision of the energy component estimates is vital for designing optimal experiments and for maximally exploiting indirect calorimetry datasets.

We here discuss the computational procedures that can be used for inferring energy components from time-dependent indirect calorimetry data and we discuss the various factors that affect the precision and performance of these methods. To provide practical and quantitative insight into how each approach works and what their differences are, we analysed high resolution datasets of mice and rats and investigated the effect of several experimental settings.

ESTIMATION OF ENERGY COMPONENTS

GENERAL PRINCIPLES

TEE in animals and man can be subdivided into four main components: the basal metabolic rate (BMR), which is the minimum amount of energy that is needed by the body to sustain vital functions, the activity related energy expenditure (AEE), which is the energy associated with muscular work, the thermic effect of food (TEF), which is the energy associated with the digestion, absorption and storage of food, and the energy expenditure due to thermoregulation (TR), which is the additional heat generated to keep the body at a constant temperature (Blaxter, 1989; Bursztein et al., 1989; Cannon and Nedergaard, 2011).

Employing calorimetric techniques one can have direct access only to the TEE, and experimental interventions and dedicated computational techniques are required to disentangle its components. The set of procedures that is involved with decomposing TEE is referred to as component analysis. For indirect calorimetry data obtained in freely moving subjects, the first step of component analysis consists of separating the activity related energy component from the resting component.

$$\begin{aligned} \mathrm{TEE}(t) &= \mathrm{AEE}(t) + \underbrace{\mathrm{BMR}(t) + \mathrm{TEF}(t) + \mathrm{TR}(t)}_{} \\ &= \mathrm{AEE}(t) \quad + \qquad\qquad \mathrm{RMR}(t) \end{aligned} \tag{1}$$

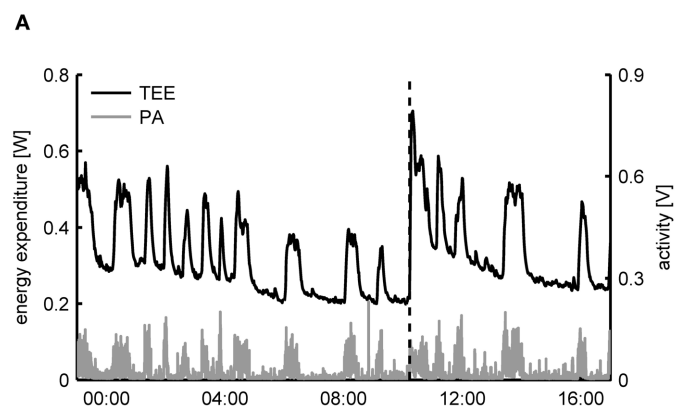
The energy component not involved in PA is commonly referred to as the resting metabolic rate (RMR) (Blaxter, 1989; Bursztein et al., 1989), or the background metabolism (Even et al., 1991), and comprises the BMR, TEF, and energy expenditure due to TR. Since each energy component can show large variations in intensity during the course of a day or over longer periods, the time dependence (t) is explicitly stated in (1).

For freely moving animals the decomposition of TEE into an activity related and resting component can be achieved by exploiting the time correlation that exists between recorded activity patterns and the TEE. As an example, in **Figure 1A** the time-dependent TEE and the activity pattern of a mouse are displayed, clearly showing that the fast increases in TEE overlap with the onset of periods of PA. Importantly, since the changes in TEE due to activity are much quicker than the time variation in the other components, it becomes statistically feasible to identify the activity related component in the TEE. More specifically, the basic assumption of TEE decomposition is that—for successive measurements taken in the same subject—the intensity of PA has a strong linear correlation with the AEE

$$\mathrm{AEE}(t) = \mathrm{CCA}(t) \cdot \mathrm{PA}(t) + e(t) \tag{2}$$

with CCA the caloric cost of activity, which is the relative amount of energy needed for an activity bout, and e the residual energy expenditure, which is assumed to be a normally distributed random variable with zero mean. Since different types of activity can have different caloric costs (Meyer and Guillot, 1986; Heglund and Taylor, 1988), the CCA is time dependent. The CCA is an interesting variable in itself and has been suggested as a measure of the mechanical efficiency of an organism and the coupling between oxygen consumption and ATP production in muscles (Even and Nadkarni, 2012). It is important to note though that the estimate of the CCA also depends on the type of activity sensor, and therefore the validity of biological interpretations of the CCA ultimately depends on the quality of the activity sensor and on how accurately it quantifies the intensity of activity.

The first step in component analysis revolves around estimating the CCA, which permits determining the time-dependent RMR by Equations (1) and (2). Computational techniques for estimating CCA, AEE, and RMR from indirect calorimetry data will be discussed in the next section. The second step of component analysis consists of decomposing RMR further with experimental interventions. The energy component related to TR can be removed by housing animals in thermoneutral conditions,



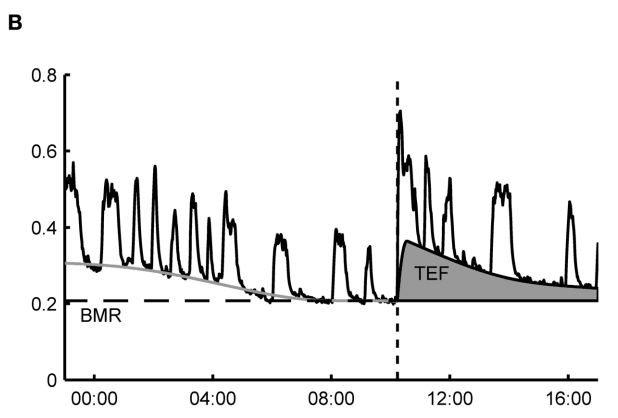


FIGURE 1 | (A) Typical dataset of total energy expenditure (TEE) and physical activity (PA) of a mouse measured in a metabolic cage employing open flow indirect calorimetry. From the time patterns in TEE and PA it can be clearly seen that the fast fluctuations in the TEE are correlated with the PA. At 10:00 (dashed line) a single meal was given, after which TEE increased due to the thermic effect of food (TEF). **(B)** Decomposition of TEE consists of exploiting the correlation between the fast time fluctuations in TEE and PA to infer the resting metabolic rate (RMR) and the activity related energy expenditure in a time dynamic fashion. The basal metabolic rate (BMR) is calculated here as the RMR after an overnight fast and the TEF is quantified as the increase in RMR above the BMR after feeding.

which for mice lie around 30°C and for rats 28°C (Cannon and Nedergaard, 2011; Tschöp et al., 2011; Even and Nadkarni, 2012). Lately, it has been argued that thermoneutrality should be the standard condition for evaluating energy expenditure in rodents rather than at typical ambient temperatures of 20–23°C (Cannon and Nedergaard, 2011; Even and Nadkarni, 2012). The first reason is that under these conditions rodents represent a better model for obesity in humans, since humans normally live under thermoneutral conditions because of clothing. Secondly, below thermoneutral temperatures non-shivering thermogenesis is a variable energy component that can compensate for—and hence mask—variation in other components such as the AEE (Humphries and Careau, 2011; Virtue et al., 2012).

The RMR at thermoneutrality, or RMRt (Speakman et al., 2004), consists of the BMR and TEF. The BMR can be determined by estimating the RMRt after a period of fasting to remove the TEF component. There is some debate on the optimal length of the fasting period to reliably assess BMR in rodents. Some researchers advise a relatively short period of 4–5 h (Speakman, 2013), in order to prevent the animal to go into a state of torpor which decreases the BMR (Hudson and Scott, 1979), while others use a period of overnight fasting (typically more than 12 h) to be sure that the TEF component is completely removed from the RMRt (Even and Nadkarni, 2012). We will abstain here from advocating either approach but simply state that for the communication of experimental results it is important to clearly declare how BMR was measured and how long animals were fasted in order to make the comparison of results from different studies possible.

After a period of fasting the TEF can be determined by presenting a single meal and subsequently calculate the area under the curve of the increase in RMRt above the BMR (**Figure 1B**) (Even et al., 1994). The same protocol is used for determining TEF in humans, with the exception that energy expenditure is then determined after the consumption of a meal in a resting, supine posture, which makes the correction for activity unnecessary (Reed and Hill, 1996). An alternative approach for determining the TEF that allows subjects to freely move and consume multiple meals during the day is to use regression analysis to simultaneously estimate AEE and TEF from the time variations in the TEE (Van Milgen et al., 1997; Van Milgen and Noblet, 2000). However, since the dynamic response of energy expenditure on food intake is much slower than PA, statistically it is only possible to discern TEF from the RMR using regression if meals are consumed separated by large enough time intervals—typically around three meals per day—such that sufficient time variation in the TEE is caused by food intake. As a consequence, this approach cannot be applied to rodents that are given *ad libitum* access to food, as then moments of food intake will occur with a high frequency (Moran, 2003).

It is important to note that except for a transient increase in energy expenditure, food intake can also have additional effects on energy metabolism. For instance, as was shown by Feldmann et al. (2009), adrenergic thermogenesis is increased in wild type mice when put on a high fat diet under thermoneutral conditions. Disentangling the effect of diet composition on energy expenditure from the TEF may be difficult in data from single animals because the two processes will overlap in time. Rather, the effects of diet composition must be assessed by comparing energy components between groups of mice that have been put on different diets.

An interesting extension of component analysis is to decompose the time-dependent oxygen consumption and carbon dioxide production separately (Van Milgen et al., 1997). In this way it becomes possible to calculate the respiratory quotient (RQ) related to activity and resting metabolism, which permits to investigate fuel selection in greater detail. For instance, from the dynamic response of the activity and resting RQ after food intake or other metabolic challenges, insight can be gained into the regulation of substrate oxidation and metabolic flexibility (Kelley and Mandarino, 2000; Even and Nadkarni, 2012).

METHODS

ALIGNMENT OF ACTIVITY AND ENERGY EXPENDITURE DATA

Over the years several computational methods have been proposed for estimating the activity related part of TEE. These methods are based on assumption (2), namely on that there exists a strong correlation between the time patterns of AEE and PA. However, the correlation between the raw time sequences of the PA and energy expenditure is usually very poor and preprocessing of the data is needed to maximize their correlation and make TEE decomposition possible. The most important step in data preprocessing is to take into account the fact that the time pattern in the respiratory exchange is dampened due to gas mixing in the metabolic chamber, while activity measurements are instantaneous (Arch et al., 2006; Lighton, 2008). Modeling the chamber as a linear compartment, the effect of gas mixing is mathematically described by the impulse response function $h(t)$

$$h(t) = \begin{cases} \frac{1}{\tau} e^{-\frac{t-\tau_{\text{delay}}}{\tau}} & t \geq \tau_{\text{delay}} \\ 0 & t < \tau_{\text{delay}} \end{cases} \quad (3)$$

with τ the washout time of the chamber, which in the case of ideal mixing is equal to the chamber volume divided by the air flow, and τ_{delay} the delay introduced by the tubing and gas dryers that are situated between the outlet of the chamber and the gas sensors. In practice τ may be found 5–10% lower than its theoretic value—that is, the ratio of the chamber volume and air flow—because of dead spaces in the chamber. A more precise model of gas diffusion also takes into account the gas diffusion inside the body, which extends (3) to a two compartment model; for details, see Van Klinken et al. (2012). However, since the washout time induced by the chamber is typically much larger than that of the animal (often by a factor of least 10) the single compartment model normally gives a reasonable approximation.

Given τ and τ_{delay}, there are two possible solutions to align the raw PA and TEE time sequences. The first approach consists of applying the impulse response $h(t)$ on the activity data and thus induce the same deformation as on the gas exchange. This approach is computationally the simplest and involves the application of a so-called infinite impulse response filter to the activity data. For a single compartment model and a sequence of activity

data PA(i) measured with sample time T, the diffusion corrected activity PA*(i) is calculated as

$$\mathrm{PA}^*(i) = a \cdot \mathrm{PA}^*(i-1) \quad + \quad b \cdot \mathrm{PA}(i) \tag{4}$$

with i the index in the PA sequence and a and b the filter coefficients derived from the impulse invariance method (Oppenheim et al., 1999)

$$a = \exp\left(-\frac{T}{\tau}\right) \qquad b = 1 - \exp\left(-\frac{T}{\tau}\right)$$

where τ and T are expressed in the same time units. In addition, a linear shift must be applied to account for τ_{delay}. This approach works well for both high and low resolution data, but requires the washout time τ to be relatively small such that the fast variations in TEE due to activity are not dampened to the extent that the correlation between TEE and PA is lost. Alternatively, if τ is large, as is the case for human metabolic chambers or those for large mammals, the instantaneous TEE needs to be calculated, which is a mathematical procedure called deconvolution (Arch et al., 2006; Lighton, 2008; Tokuyama et al., 2009). Deconvolution of the TEE time series, though, is a more complicated procedure because it is very susceptible to sensor noise and therefore requires the application of filtering techniques to attenuate the effect of noise. Consequently, deconvolution can only be applied on frequently sampled data, as this increases the ability of filters to get rid of sensor noise while leaving the original signal intact.

LINEARISATION OF ACTIVITY AND ENERGY EXPENDITURE DATA

In addition to the correction for gas mixing effects, it may sometimes be necessary to apply a non-linear transformation to the raw activity data in order to linearize the relation between activity and energy expenditure. Other mathematical functions that can be applied to the activity signal are a threshold to correct for a baseline activity signal or a kernel to smoothen noisy activity data; for details, see Van Klinken et al. (2012), supplemental text 2. Whether or not there is a need to transform activity data can be investigated by inspecting the scatter plot of the diffusion corrected activity and the TEE or the residuals from component analysis. If non-linear trends are perceivable in these plots then an appropriate function must be chosen to transform the activity data, since otherwise the linearity assumption (2) is violated and decomposition may become unstable. The best practice for selecting the free parameters of the non-linear transformation is to minimize the residual variation that results from the decomposition method. Since the same transformation must be applied to the activity data from all subjects participating in an experiment, it is important to minimize the sum of residuals from all datasets.

As an example, **Figure 2A** shows the relation between the energy expenditure and the activity signal from piezo-electric sensors measured in 11 mice. To properly overlap the data of all mice, the activity (x-axis) has been multiplied by the subject specific CCA that resulted from penalized spline (P-spline) regression. The scatter plot shows that there exists a slight non-linear relation between the activity and the energy expenditure, which is confirmed by the scatter plot of the residual energy expenditure and the activity. Moreover, the activity signal contains a non-zero baseline, which needs to be subtracted from the total activity signal to prevent the AEE estimate from being biased. Applying a threshold and an exponent of 0.6 linearizes the relation and shifts the left tail of the point cloud to the origin (**Figure 2B**).

An alternative approach for aligning TEE and PA data is to bin both signals into intervals of a given length; e.g., see Nonogaki et al. (2003), Bjursell et al. (2008), and Virtue et al. (2012). Technically this procedure corresponds to the application of a low pass filter to both signals, which makes sense because the low frequency TEE and PA signals correlate better in time. Nevertheless, the resulting correlation is less strong as when applying the linear compartment model, as is illustrated in **Figure 2C**. Consequently, using the linear compartment model (i.e., Equation 4) should be the preferred approach for aligning TEE and PA data for the purpose of regression analysis, since this gives a smaller residual error and therefore more accurate regression estimates.

REGRESSION ANALYSIS

The classical technique for estimating CCA and decomposing TEE into an activity and resting related part is by performing linear regression of TEE against PA for each subject separately (Ravussin et al., 1986; Nonogaki et al., 2003; Kumahara et al., 2004; Bjursell et al., 2008). Using this approach, the intercept of the regression line with the y-axis corresponds to the average RMR and the slope to the CCA. The advantage of this method is that it is relatively simple to use and can be executed by standard (spreadsheet) software packages. However, one of the main disadvantages is that it does not take into account the time variation that is present in the resting energy expenditure, which increases the amount of uncertainty in the CCA and RMR estimates and can introduce a bias into the results.

As was first put forward by Brown and colleagues (1991), it is possible to take into account the time variation of the RMR by modeling the RMR with a more complex, time-dependent function. Recently we proposed a solution for regression based component analysis in which the time-dependent RMR is modeled with P-spline functions (Van Klinken et al., 2012). This makes it possible to obtain an estimate of the time variation in the RMR, but also increases the accuracy with which the CCA and AEE are estimated. In addition, this method has demonstrated to be relatively robust to low sample frequencies and noisy activity measurements, which is important when performing component analysis on data generated by multiplexed systems.

When using P-spline regression for component analysis, first the number of knots has to be chosen in the spline function that models the time variation in the RMR. This parameter determines how quickly the RMR can vary with time. In our earlier study we showed that with approximately $2k$ knots per day, frequency components in the RMR of up to k day^{-1} can be estimated (Van Klinken et al., 2012). Importantly, slow time variations in the RMR can be estimated with a higher robustness than fast time variations, but they will also introduce a bias when RMR changes quickly. In practice, inspection of the time-dependent plots of the TEE, PA and fitted RMR is needed for determining the right amount of knots.

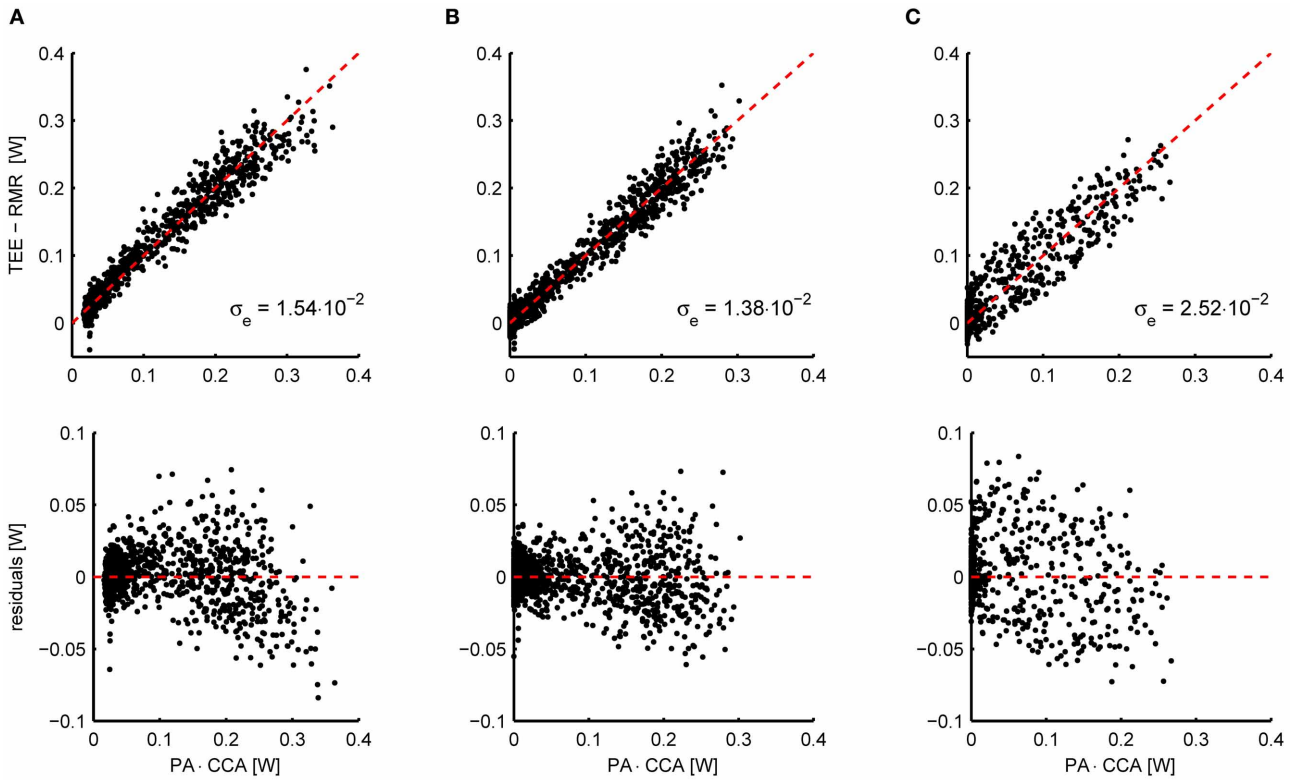


FIGURE 2 | (A) Scatterplot of the activity signal from piezo-electric sensors corrected for gas mixing and the total energy expenditure (TEE) minus the resting metabolic rate (RMR) in mice ($n = 11$) (top) and the residual energy expenditure TEE – RMR – AEE (bottom). The AEE and RMR were calculated with P-spline regression. The relation between energy expenditure and activity is slightly curved, which is accentuated when plotting the residuals of the fitted P-spline model against the activity. Moreover, a non-zero baseline is present in the activity signal. **(B)** Applying a threshold to correct for the baseline activity signal and an exponent of 0.6, the relation between activity and energy expenditure is sufficiently linearized. As a result of the preprocessing, the standard deviation in the residuals is reduced from $\sigma_e = 1.54 \times 10^{-2}$ W to $\sigma_e = 1.38 \times 10^{-2}$ W. The preprocessing parameters were fitted by minimizing the residuals of the P-spline model summed over all mice. **(C)** Instead of applying the linear compartment model to the PA signal to increase the correlation between the TEE and PA time series, they can also be binned in a given time interval (here 15 min). Although this procedure increases the correlation between both signals with respect to the untransformed data, binning does give inferior results when compared to the linear compartment model **(B)**.

KALMAN FILTERING

An alternative approach to regression analysis is to decompose TEE using Kalman filtering, as was originally proposed by Even and Nicolaidis (1984) and Even et al. (1991). The basic assumption of this method is that the RMR and CCA can be modeled as Gaussian random processes that vary in time. The relation between the RMR, CCA, AEE, and TEE and the effects of gas mixing are expressed in a state space model, which permits the estimation of the state variables by means of Kalman filtering. Kalman filtering is a numerical filtering technique that works by continuously predicting the future state of the system and then correcting the prediction with the measured data. This procedure relies on the fact that data has been sampled with a relatively high frequency, and it is therefore best suited for analysing data generated by single channel indirect calorimetry systems with continuous data acquisition (Even and Nadkarni, 2012). Since the state space model already includes the effects of gas mixing on the measured respiratory exchange, the data must not be corrected for these effects as part of preprocessing. Importantly, the performance of the Kalman filter depends on the choice of filter parameters, which must be set in advance by the user by tuning the filter.

MINIMAL METABOLIC RATE

A popular approach for estimating the BMR from indirect calorimetry datasets in animals is to take the minimum energy expenditure that is reached after a period of fasting (Selman et al., 2001; Speakman, 2013). Since random fluctuations in the resting energy expenditure can induce a downward bias in the BMR estimate obtained in this way, normally the minimal energy expenditure is taken averaged over a short, e.g., 5 min, interval. The advantage of this method is that it does not involve correlating PA and TEE—activity does not even have to be measured. A potential downside, however, is that the BMR estimate is influenced by several experimental settings. Most importantly, the registered minimum metabolic rate depends on the duration of the interval over which is sampled and on the sampling frequency, since sampling for a longer period or with a higher frequency

will increase the likelihood of finding a low measurement by chance (Cooper and Withers, 2010). Also, the washout time of the metabolic chamber influences the BMR estimate, since if the measured respiratory exchange needs too long to return to base level after an activity bout has occurred then the BMR estimate will be biased upward. Finally, the measured minimum metabolic rate depends on the activity pattern of the animal, since animals that rest more often or have longer pauses will exhibit more minima.

COMPARISON OF METHODS

EXPERIMENTAL DATA

To make a baseline comparison between the results that are obtained by decomposition methods, we tested Kalman filtering and P-spline regression on high resolution datasets of mice ($n = 11$) and rats ($n = 47$) that have been made available by P. C. Even. In short, for each animal respiratory exchange was measured during 24 h with a sample time of either 2 or 5 s. Mice were housed at 30°C and rats at 28°C under a standard 12 h:12 h light/dark cycle. Activity was measured with piezo-electric force transducers and was averaged over periods equal to the sample time. Animals were placed in the metabolic chamber at 18:00 with water but no food and were fasted overnight, after which they were fed a single meal between 9:00 and 10:00. Weir's equation was used to calculate energy expenditure from oxygen consumption and carbon dioxide production rates (Weir, 1949).

We performed P-spline regression to estimate the time-dependent activity related and resting respiratory exchange using a spline function containing 8 knots. Also the CCA was allowed to vary in time and was modeled using a spline function with 16 knots. Because the meal induced a very rapid rise in the energy expenditure, we applied P-spline regression separately on the pre-feeding data and the data obtained 20 min after food intake; the intermediate 20 min interval was interpolated using a 2nd order polynomial function. To increase robustness in the RMR estimate, weighed regression was used where measurements were given a weight of 0.2 during periods of activity and a weight of 1.0 during resting periods. The smoothing parameter λ was estimated automatically using the generalized cross validation criterion as previously reported (Van Klinken et al., 2012) and was subsequently averaged separately for mice and rats.

Kalman filtering was performed on the data under two different conditions: with the filter parameters fitted to minimize the difference between the time-dependent RMR estimates of the Kalman filter and the P-spline model, and with the filter parameters manually tuned without taking the results of the P-spline model into account. The initial estimate of the state vector was obtained by linear regression; the initial 4 h of data were used for the Kalman filter to converge and were not used in the further analysis. To account for the rapid change in energy metabolism after feeding, the process noise variance associated with the CCA was increased by a factor of 10 for a window of 120 min after feeding. For both the Kalman filter and the P-spline regression approach a threshold and non-linear transform was applied to the raw activity data as explained in **Figure 2**. All calculations were performed in MATLAB (The MathWorks).

COMPARISON OF THE TIME-DEPENDENT RMR

From a visual comparison of the time-dependent RMR estimates it followed that even in the case that the Kalman filter was tuned manually there was a good consensus between both methods for most subjects (**Figure 3**). A general difference that we observed was that the RMR estimated by the Kalman filter exhibited more variation than the result of P-spline regression, especially during periods of activity. This divergence can be explained by the way how both methods operate: the Kalman filter assumes that there is a small residual signal and attributes fast fluctuations to the RMR whereas the P-spline regression model assumes that the RMR varies slowly in time and attributes most of the fast fluctuations to the residuals.

Some care has to be taken when interpreting the fast, non-activity related, time fluctuations in the respiratory exchange as they can have both biological and technical origins. Fast fluctuations of a biological origin are those associated with the sudden increases in energy expenditure due to (endogenous) nervous and hormonal changes. In contrast, variations that are characterized by a negative spike and a subsequent more blunted positive

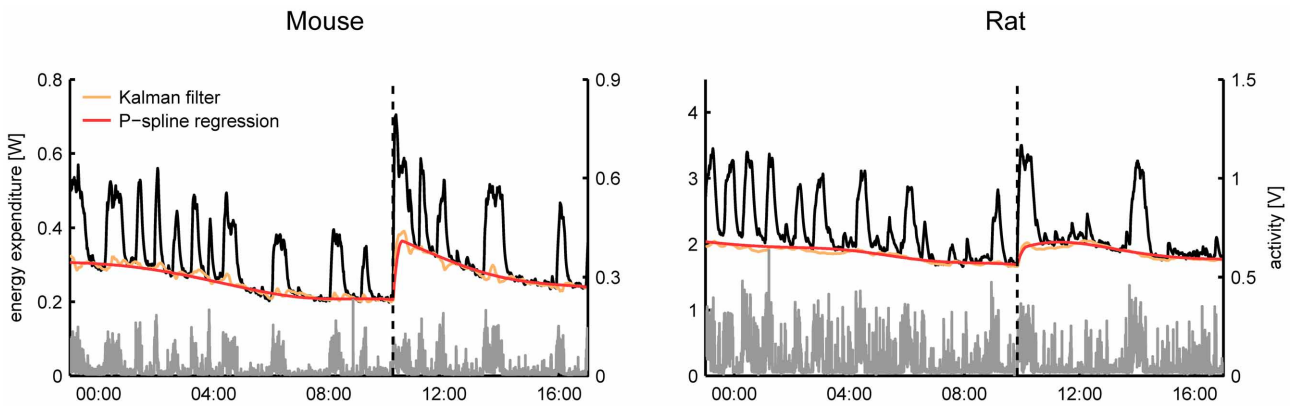


FIGURE 3 | Time plots of the total energy expenditure, activity and estimated RMR of a mouse and a rat using P-spline regression and Kalman filtering with manually tuned parameters. On average there is a good correspondence between the results from both methods, with the Kalman filter showing slightly more variation in the RMR estimate than the P-spline method.

peak are indicative of short periods of apnea and therefore reflect respiratory dynamics rather than true changes in energy expenditure (Speakman, 2013). Importantly, fluctuations observed in the non-activity energy expenditure during activity bouts are often caused by the non-perfect match between the registered activity and the energy expenditure—e.g., due to noise in the activity sensor or to the non-ideal mixing of air in the metabolic chamber—and can therefore be technical in nature.

COMPARISON OF INFERRED METABOLIC PARAMETERS

In addition to comparing the time-dependent results we investigated how well the estimates of a number of metabolic parameters corresponded between the Kalman filter and P-spline regression. In detail, we derived the following metabolic parameters from the time-dependent activity and resting VO_2 and VCO_2 of each method: the BMR, calculated as the average RMR over a period of 90 min prior to feeding; the TEF, calculated as the area under the curve in RMR above the BMR after feeding for a period of 5 h; the AEE and CCA during the fasting period; the resting (activity) RQ during fasting, calculated as the total resting (activity) VCO_2 produced divided by the total resting (activity) VO_2 consumed during a 5 h period prior to feeding; and the increase in resting (activity) RQ due to feeding, calculated as the total resting (activity) VCO_2 produced divided by the total resting (activity) VO_2 used during a 5 h period after feeding, *minus* the fasting resting (activity) RQ.

Figure 4 shows Bland Altman plots for each of the eight metabolic parameters that were estimated from the results of P-spline regression and Kalman filtering with parameters fitted to minimize the difference with the time-dependent RMR of the P-spline model. It follows that there was a very high correlation between both methods for most metabolic measures, which shows that both methods can give virtually identical results on high resolution data as long as the parameters of the Kalman filter are tuned by using an external criterion. When the filter parameters were tuned manually the correlations generally dropped (**Table 1**), but were still very good for the BMR, resting RQ during fasting and the increase in resting RQ, with $R^2 > 0.95$ for both mice and rats. There was also a high agreement between the AEE estimates with R^2 around 0.97, but this dropped to $R^2 = 0.91$ for rats when manually tuning the filter. The correlation was also strong for the TEF with R^2 between 0.87 and 0.91, while for the CCA the correlation was considerably lower with R^2 between 0.59 and 0.80. The reason why the R^2 of the CCA is much lower than that of the AEE is that the CCA varies less between animals than the AEE: the coefficient of variation of the CCA is 0.09 in mice and 0.08 in rats whereas for the AEE it is 0.27 in mice and 0.20 in rats. Consequently, the denominator of the fraction of unexplained variance is relatively smaller in the CCA, which explains why the R^2 is lower. The correlation of the activity related RQ during fasting and the increase after feeding in mice dropped considerably when manually tuning the filter, which shows that this measure is very sensitive to the choice of filter parameters. For rats the correlation was worse than for mice, which was probably caused by the fact that rats were overall much less active after prolonged fasting and after refeeding, inducing thus a larger degree of uncertainty in the activity RQ estimates.

The results in **Figure 4** show that on high resolution data there is an overall good agreement between the metabolic parameters as determined from the decomposition results of the Kalman filter and P-spline regression method, except for the activity related RQ in rats. The differences between the metabolic parameter estimates are due to the different assumptions that are at the base of both methods, most notably regarding how the time variation in the RMR and CCA is modeled: the Kalman filter uses random Gaussian processes, which exhibit very fast changes, while the P-spline method models the RMR and CCA with spline functions that vary slowly in time. This difference is magnified during periods of activity (**Figure 3**), which explains why the metabolic measures related to resting metabolism are more accurate than those related to activity.

MEASUREMENT OF BMR

Since the RMR is relatively stable when it has converged to the BMR after a period of fasting, the BMR can also be estimated by methods that assume a constant resting energy expenditure. We compared the BMR estimate of the Kalman filter and P-spline regression with the minimal energy expenditure (**Figures 5A,B**) and with the BMR estimated by linear regression using the linear compartment model for gas diffusion effects (**Figures 5C,D**). Both methods were applied on the data obtained during the 90 min period prior to feeding. To reduce the downward bias caused by randomly occurring dips in the RMR, we calculated the minimum averaged energy expenditure for a 5 min window.

Both the minimal EE as the BMR estimate from linear regression had a good correspondence with that of Kalman filtering and P-spline regression ($R^2 > 0.94$). However, the minimal EE was biased downwards with respect to the BMR estimate of the Kalman filter and P-spline regression, which means that additional postprocessing of the results to correct for the bias is necessary when using this method. In comparison, the BMR estimate by linear regression was not biased and also strongly correlated with the result of the Kalman filter and P-spline regression, suggesting that linear regression is an adequate alternative for estimating the BMR when the RMR has stabilized. Estimating the BMR with linear regression while binning PA and TEE in 15 min intervals gave worse results than the linear compartment model (**Figures 5E,F**). The larger divergence from the Kalman filter and P-spline regression estimates can be explained both by the larger residual variance obtained by binning (**Figure 2C**) and by the larger uncertainty that is present in the BMR estimate because regression was based on less data points.

INFLUENCE OF EXPERIMENTAL VARIABLES

The accuracy with which the computational methods described in the previous section are able to partition the TEE into a time sequence of the activity and resting energy component depends on a number of properties and settings of the indirect calorimetry system. Most importantly, these are the sampling frequency with which the respiratory exchange and activity are measured, the type and accuracy of the activity sensor, and the size of the chamber in proportion to the flow rate. Since the estimates of the TEF, BMR, and activity and resting RQ are derived from the result of the decomposition method, it is important to know how the

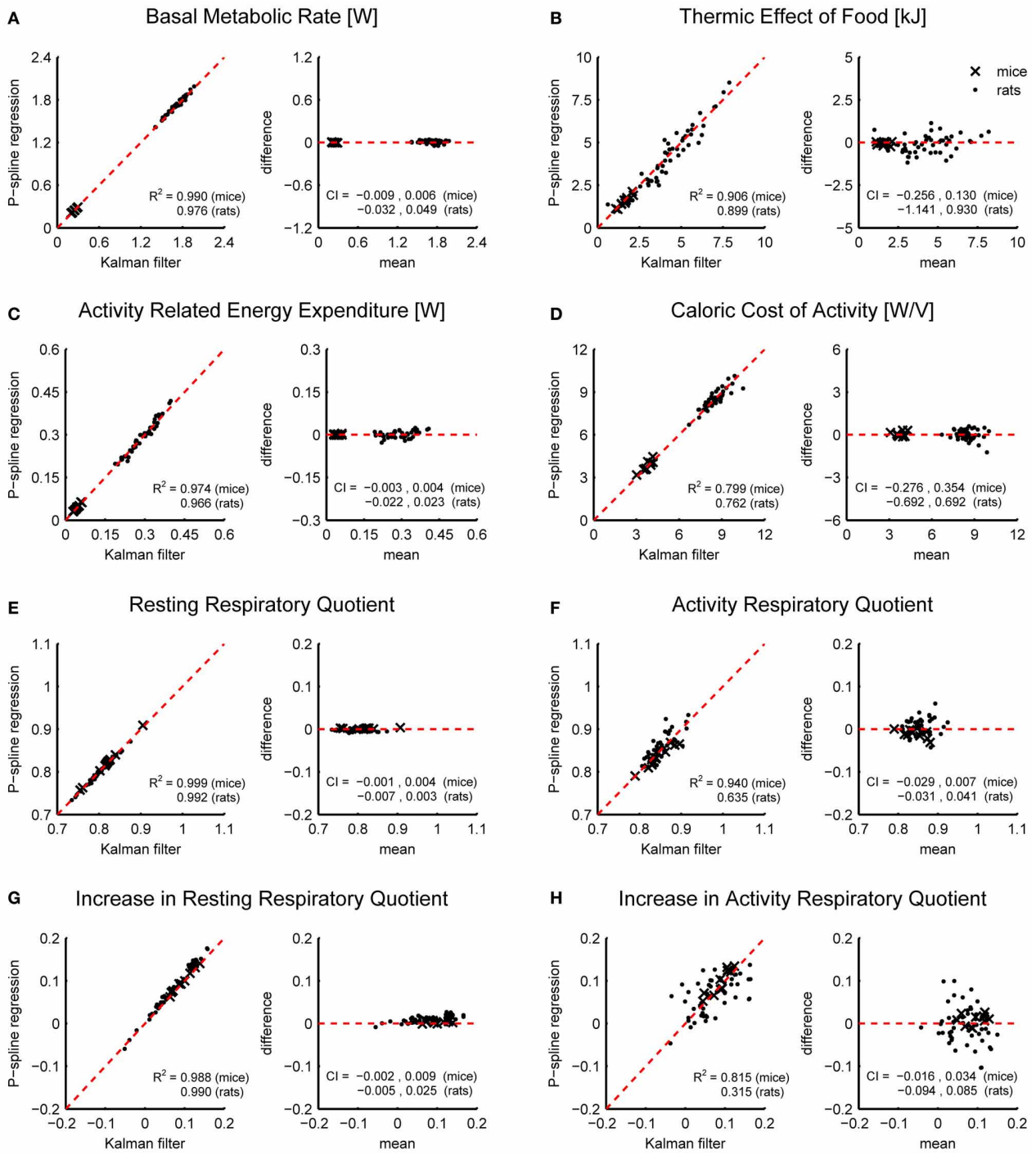


FIGURE 4 | Correspondence of metabolic parameter estimates from indirect calorimetry datasets of mice and rats using Kalman filtering and P-spline regression. Bland Altman plots are shown of the BMR **(A)**, TEF **(B)**, AEE **(C)**, CCA **(D)**, resting RQ during fasting **(E)**, activity RQ during fasting **(F)**, increase in resting RQ after feeding **(G)**, increase in activity RQ after feeding **(H)**. The coefficient of determination (R^2) and 95% confidence interval (CI) are shown separately for mice and rats.

experimental settings affect a method's robustness and, in turn, that of the inferred metabolic measures.

We here tested how the eight metabolic measures presented in the last section are affected by changes in the sampling resolution, the accuracy of the activity sensor, and the system's washout time for the existing experimental data. For each case the effects of a change in the experimental setting was simulated on the existing data, and subsequently component analysis was performed on the

Table 1 | The coefficient of determination (R^2) of the correlation for metabolic measures based on the results of P-spline regression model and of the Kalman filter with automatically and manually tuned filter parameters.

	Mice		Rats	
	Automatic	**Manual**	**Automatic**	**Manual**
BMR	0.990	0.987	0.976	0.958
TEF	0.906	0.911	0.899	0.866
AEE	0.974	0.975	0.966	0.908
CCA	0.799	0.777	0.762	0.589
Resting RQ	0.999	0.995	0.992	0.978
Activity RQ	0.940	0.878	0.635	0.372
Δ Resting RQ	0.988	0.962	0.990	0.987
Δ Activity RQ	0.815	0.373	0.315	0.188

Automatically tuned parameters of the Kalman filter were fitted as to minimize the difference with the time-dependent RMR estimate of the P-spline regression model, resulting therefore in energy component estimates that are as close to the result of the P-spline regression model as possible. In contrast, manually tuned parameters were based on visual criteria and did not include prior knowledge of the results of P-spline regression. The agreement between metabolic measure estimates is typically worse when the Kalman filter is manually tuned, though correspondence is still good for the BMR, TEF, AEE, and the resting RQ. For the CCA the correlation is less strong, while for the activity RQ during fasting in rats and the increase in activity RQ after feeding the correlation is very poor, showing that these measures are very sensitive to the specific method that is used and to how the parameters were tuned.

new data; for details regarding the simulation procedure, see the respective sections. The estimate of each parameter on the new data was then compared with its basal estimate and used to calculate the mean squared error (MSE) relative to the total variance for the Kalman filter and P-spline regression:

$$\text{relative MSE}(x) = \frac{\sum_i \left(x_i - x_{ii}^{\text{basal}}\right)^2}{\sum_i \left(x_{ii}^{\text{basal}} - \bar{x}_i^{\text{basal}}\right)^2} \cdot 100\%$$

with x_i the estimate of metabolic measure x for subject i on the new data and x_i^{basal} the estimate on the original data. Importantly, the relative MSE approximates the unexplained variance $1 - R^2$, except that it also penalizes a difference in the mean estimates; this property assists in the interpretation of the relative MSE since it can be directly related to the R^2. It should also be noted that since the relative MSE is auto-referenced (i.e., to the basal estimate) it does not include systemic errors due to drift in the CO_2 and O_2 sensors or the mass flow controller.

Since the choice of the filter parameters became sub-optimal when applying the Kalman filter on the data with the simulated experimental changes, we adjusted these parameters as to minimize the total MSE of the Kalman filter. No parameters were changed for the P-spline regression method on the new data.

SAMPLING RESOLUTION

The sampling frequency with which the respiratory exchange can be measured depends on the type of indirect calorimetry system. For single channel systems a set of O_2 and CO_2 analysers continuously measure the respiratory exchange in a single chamber, and therefore there are no restraints on the sampling frequency other than technical limitations related to data storage and data handling. Gas sensors, though, are rather expensive and therefore most commercial manufacturers of indirect calorimetry systems have opted for a multiplexed design where a single set of gas sensors measure the air from multiple chambers in succession. This comes at the cost of reducing the time resolution that can be attained, because now the tubing between each chamber and the sensors needs to be purged every time a new measurement is taken.

Sampling frequency can have a huge impact on the performance of decomposition algorithms and also puts a limit on the amount of detail that can be attained in the estimate of the time-dependent RMR. In fact, recently it has been questioned whether detailed component analysis is even possible on data that has been generated by multichannel systems (Even and Nadkarni, 2012). Hence, it is important to quantify the influence sampling frequency has on the precision with which metabolic parameters are estimated, in order to understand what can be achieved with a certain indirect calorimetry system or experimental design. We therefore performed TEE decomposition on a range of downsampled versions of the original high resolution VO_2 and VCO_2 time sequences and compared the estimates of the eight metabolic parameters with their basal estimates. To simulate a dataset with a sample time of T seconds we took every N-th measurement of the VO_2 and VCO_2 while discarding the rest, where N is defined as T divided by the sample time of the original dataset. Since there are typically no limitations on the sampling frequency with which activity can be measured, we did not perform downsampling of the activity data.

Figure 6 shows the relative MSE of Kalman filtering and P-spline regression in estimating each metabolic parameter for a sample time ranging from 5 s to 20 min. In general, the deviance of each estimated parameter from its basal estimate grows with increasing sample time. The BMR and resting RQ during fasting seem to be the parameters that are most robust against increasing sample time. At a sample time of $T = 9$ min, the MSE of the BMR estimated with P-spline regression is 0.3% for mice and rats, and the MSE of the resting RQ during fasting lies around 0.2%, showing that for these measures there is an exceptionally high correlation of $R^2 > 0.997$ with the basal estimate for $T = 9$ min. The MSE of the increase in resting RQ after feeding in mice is with 0.9% larger than that of the resting RQ during fasting, but still very robust. Surprisingly, also the AEE and TEF can still be estimated with a decent amount of accuracy at $T = 9$ min: the AEE has an MSE of 2.2% (mice) and 1.4% (rats) and the TEF has an MSE of 2.2% (mice) and 1.1% (rats). In comparison, the other measures are more susceptible to increasing sample times. At $T = 9$ min, the MSE of the CCA is 20.3% (mice) and 11.4% (rats) and the MSE of the activity RQ during fasting is 19.7% (mice) and 7.6% (rats). These errors correspond to an R^2 with the basal estimate in the range of 0.8–0.9, which clearly show that these parameters cannot be measured with a high degree of accuracy in data coming from multichannel systems.

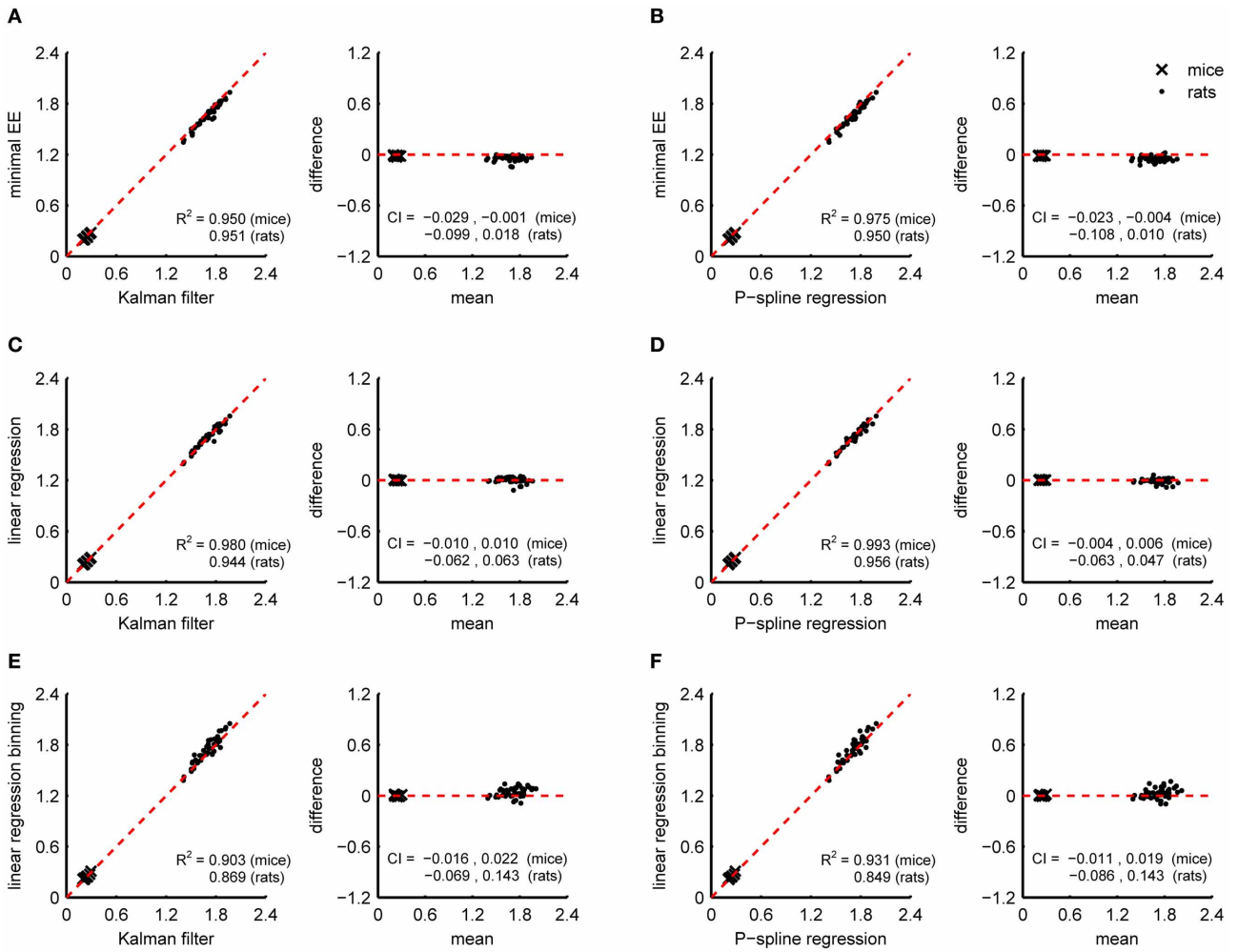


FIGURE 5 | Correspondence of BMR estimates from indirect calorimetry datasets of mice and rats with the minimal energy expenditure approach, linear regression, Kalman filtering and P-spline regression. Bland Altman plots are shown of BMR estimates of Kalman filtering and P-spline regression against minimal EE **(A,B)**, linear regression using a linear compartment model to account for gas mixing effects **(C,D)**, and linear regression using binning **(E,F)**. The coefficient of determination (R^2) and 95% confidence interval (CI) are shown separately for mice and rats.

These results show that, in general, the measures calculated from the resting respiratory exchange are less sensitive to decreasing sample rates than those related to activity metabolism. The larger sensitivity of the activity related measures, but also the TEF, is mostly due to the fact that their calculation explicitly depends on the time-dependency of the AEE and RMR estimates, and therefore suffer more heavily from the loss in time resolution. The difference in the robustness between the AEE and CCA is due to the fact that there is a much smaller basal variance in the CCA, which makes the denominator of the relative MSE smaller.

In comparison, the estimates of the metabolic parameters based on the decomposition of the Kalman filter are less robust to lower sample rates, in both mice and rats. We have found the same result in our earlier study, where we compared the performance of both methods on simulated data (Van Klinken et al., 2012). We think that the main cause for the difference in robustness is that, by its design, the Kalman filter estimates the AEE and RMR at a single time point using only a few past measurements whereas the P-spline model takes into account a larger set of local measurements which ensures more stable estimates. In addition, P-spline regression first applies the linear compartment model on the high resolution activity data and then resamples it to the measurement times of the respiratory exchange, which yields a much better correlation between the PA and TEE than when the activity data is first downsampled and then the gas mixing effects are applied, which is what occurs in the Kalman filter.

It is important to note that in our analysis we did not include the effect of additional measurement errors in the O_2 and CO_2 data that can occur due to switching between chambers by a multiplexer. When the switching between chambers occurs too fast with respect to the system configuration then old air will remain in the tubes and will affect the new measurement. In practice a choice will have to be made between a higher time resolution on one hand, and therefore more data and a better decomposition,

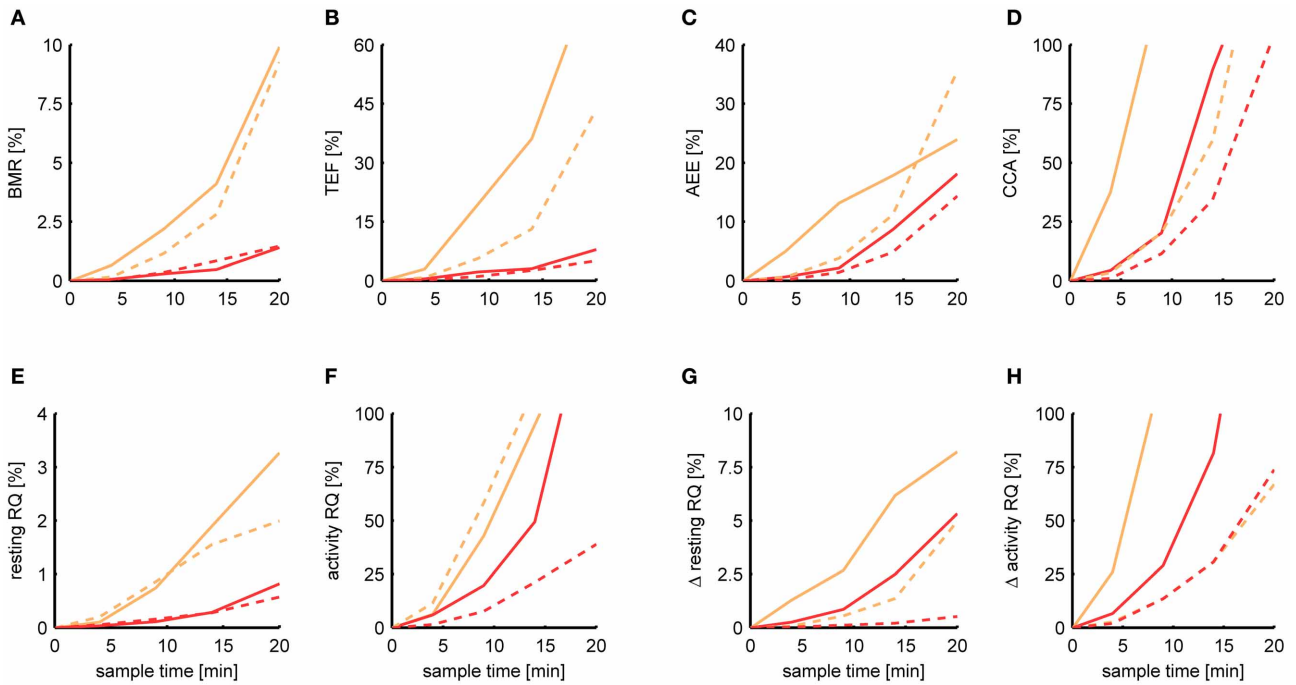


FIGURE 6 | Dependence of the estimation accuracy on the sample time. The relative mean squared error is shown of the BMR **(A)**, TEF **(B)**, AEE **(C)**, CCA **(D)**, resting RQ during fasting **(E)**, activity RQ during fasting **(F)**, increase in resting RQ after feeding **(G)**, increase in activity RQ after feeding **(H)** as estimated by the Kalman filter (orange) and P-spline regression (red) on data from mice (solid) and rats (dashed) with a sample time of 4, 9, 14, and 20 min. The relative mean squared error was calculated as the difference of the measure's estimate for a given sample time with the basal estimate at a sample time of 5 s, divided by the basal variance for that measure in mice and rats.

and a higher accuracy of O_2 and CO_2 measurements on the other. In our experience the presence of old air can be modeled with an exponential decay curve, which means that the relative error induced by old air can be quantified as a function of the decay rate and the purging time of the system. As an example, for the multiplexed indirect calorimetry system we have currently in our facility the decay rate is approximately 10 s at an excurrent flow rate of 0.4 l/min, which means that with a purging time of 60 s, 99.8% of the old air is purged, yielding a total sample time of 9 min given an 8 cage system and an empty cage for reference air.

ACTIVITY MEASUREMENTS

Several devices exist for quantifying the level of spontaneous PA in rodents, such as photocell sensors (Nonogaki et al., 2003; Bjursell et al., 2008; Kotz et al., 2008), piezo-electric force transducers (Even et al., 1991, 1994), microwave radar systems (Brown et al., 1991; Pasquali et al., 2006) and video-tracking systems (Poirrier et al., 2006). A frequently used approach in commercially available metabolic chamber systems is to measure PA as the number of infrared beam interruptions. It has been suggested, however, that infrared beam interruptions may miss out on the more subtle types of activity (Even and Nadkarni, 2012), which would make this type of sensor less suitable for TEE decomposition. Another disadvantage is that count data is inherently noisy because of the random character of beam interruption occurrences. The electrical signal generated by piezo-elements has been proposed as a more accurate alternative for measuring PA in rodents and has been suggested to correlate more tightly with the AEE and be more sensitive to small movements (Even and Nadkarni, 2012).

Since the datasets used in this study were measured only with piezo-electric force transducers, we were not able to make a direct comparison between this and other types of sensors. We therefore investigated what the effect is of the accuracy of the activity sensor on TEE decomposition in general, by adding noise to the measured activity data. Noise was simulated by multiplying the activity time sequence with the random sequence $1 + e_{PA}(t)$, where $e_{PA}(t)$ is a slowly varying random process that has been modeled with spline functions. In detail, we took $e_{PA}(t) = \Sigma_i\ \varepsilon_i\ B_i(t)$ with $B_i(t)$ the cubic B-spline basis function with randomly distributed coefficients $\varepsilon_i \sim N(0, \sigma^2)$ and with 20 knots/h. The reason to model $e_{PA}(t)$ as autocorrelated noise and not as white noise is that in the former case the situation is emulated where each activity bout has a different CCA, which is a reflection of what happens when an activity sensor is less sensitive for detecting certain kinds of activity.

Figure 7 shows the deviance of the estimated metabolic parameters for various levels of activity noise $e_{PA}(t)$. Since from the result of TEE decomposition it is not possible to discern between noise present in the activity sequence and natural

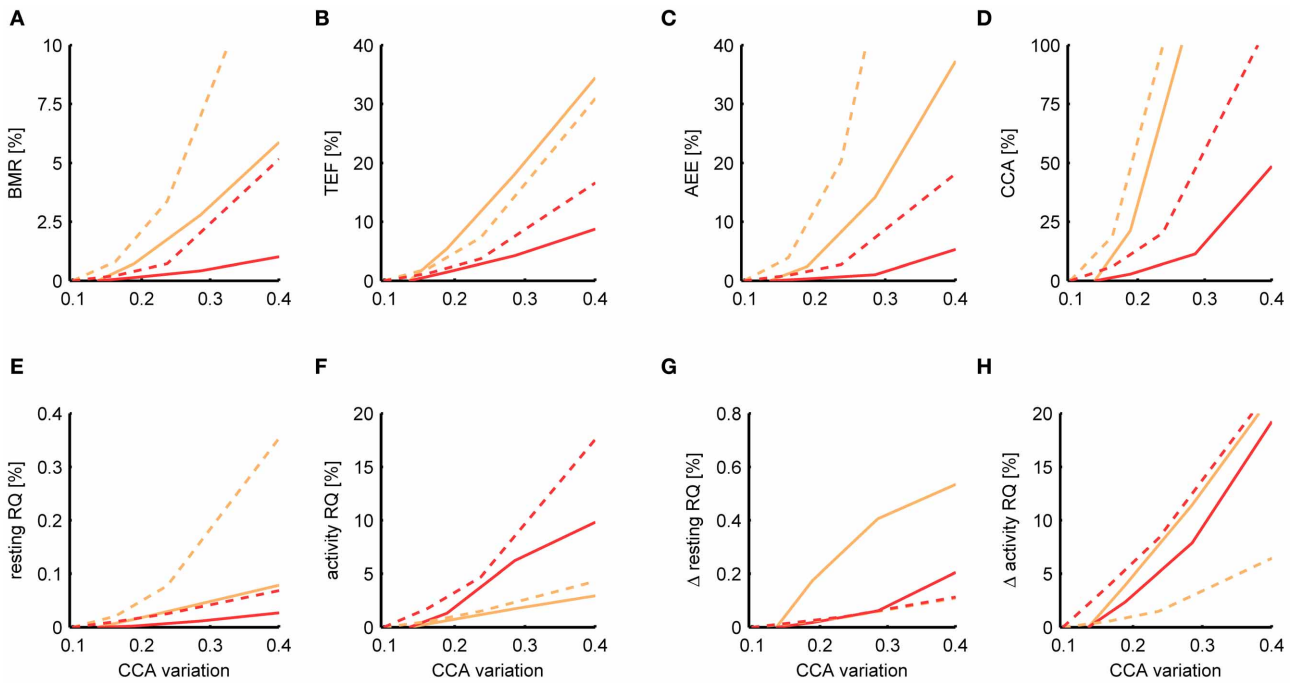


FIGURE 7 | Dependence of the estimation accuracy on the accuracy of the activity sensor. The relative mean squared error is shown of the BMR **(A)**, TEF **(B)**, AEE **(C)**, CCA **(D)**, resting RQ during fasting **(E)**, activity RQ during fasting **(F)**, increase in resting RQ after feeding **(G)**, increase in activity RQ after feeding **(H)** as estimated by the Kalman filter (orange) and P-spline regression (red) on data from mice (solid) and rats (dashed) with different levels of simulated noise on the activity data. The relative mean squared error was calculated as the difference of the measure's estimate for a given noise level with the basal estimate with no added noise, divided by the basal variance for that measure in mice and rats. The noise level (*x*-axis) is quantified as the amount of time variation σ_{CCA} in the CCA assuming a multiplicative model $CCA(t) = CCA_{const} \cdot [1 + e_{CCA}(t)]$ The baseline σ_{CCA} estimates and standard deviation are 0.136 ± 0.025 for mice and 0.099 ± 0.047 for rats.

variations in the CCA, we used the amount of variation σ_{CCA} that was present in the CCA estimate as a combined measure of the activity noise. In this way we were able to determine the effect of the simulated noise on top of the basal level of variation in CCA and could make comparisons with the basal σ_{CCA} of other activity sensors. For the time variation in the CCA we assumed a multiplicative model

$$CCA(t) = CCA_{const} \times (1 + e_{CCA}(t)) \quad (5)$$

with CCA_{const} and $e_{CCA}(t)$ the constant and time-varying part of the CCA. The variation σ_{CCA} was estimated using maximum likelihood; for details see Van Klinken et al. (2012), supplemental material 1. The basal CCA variation (mean $\pm$ *SD*) was found to be 0.136 ± 0.025 in mice and 0.099 ± 0.047 in rats. In our earlier study we measured respiratory exchange in a mouse at a high resolution using infrared beam sensors to quantify activity (Van Klinken et al., 2012); from this data we estimated σ_{CCA} to be 0.164, which lies within the 95% confidence interval of the σ_{CCA} estimate for piezo-electric sensors. Future research is needed to make a more sound comparison between these activity sensors, performing experiments in which activity is measured simultaneously with both sensors.

From **Figure 7** it follows that the estimates of the resting RQ during fasting and the increase after food intake are very robust to activity noise, having a relative MSE of less than 0.2% for all σ_{CCA}. Also the BMR was relatively robust, having a MSE of 0.4% (mice) and 0.7% (rats) at a level of twice the basal CCA variation. The AEE estimate was more sensitive to activity noise with an MSE of 1.0% (mice) and 2.7% (rats) at twice the basal CCA variation, and the TEF had an MSE of 4.2% (mice) and 3.7% (rats). The activity RQ during fasting was very sensitive to the activity noise using P-spline regression but not using the Kalman filter, which had an MSE of 1.7% (mice) and 1.5% (rats) at twice the basal σ_{CCA}. Investigating this specific observation we found that the additional uncertainty that comes with fitting the activity noise that is part of the P-splines regression algorithm had caused the larger MSE. For the other measures the P-spline regression proved to be more robust to increasing activity noise than the Kalman filter. This result is related to the use of weighed regression, which mitigates the effect of noisy activity data, and also to the choice we made for a relatively low number of knots, which prevented the P-spline model to overfit to the activity noise.

CHAMBER WASHOUT TIME

Because of the mixing process of the exhaled air with the air in the metabolic chamber, the time pattern in the O_2 consumption and CO_2 production rate measured at the chamber's outlet becomes dampened. In the ideal case, gas mixing acts as a first-order low-pass filter on the instantaneous respiratory exchange, attenuating

the high frequency variations that are due to activity. The extent of the dampening effect is directly related to the washout time τ, i.e., to the proportion of the chamber size to the rate of the air flow: all frequencies above the cutoff frequency of $f = (2\pi\tau)^{-1}$ are attenuated by the mixing process. Since component analysis ultimately relies on the time correlation between the energy expenditure and activity signal, it is important to know what effect the washout time τ has on the accuracy of the estimated components.

In order to determine the deviance from the basal estimate for varying τ, we deconvoluted the original data for the washout time of the respective experiments, and subsequently re-convoluted the data for a range of different τ's. Since re-convolution with a larger washout time implied that high frequency sensor noise was attenuated, we added white noise such that the power of frequency components above $1\,\text{min}^{-1}$ was equal for each derived dataset.

When initially comparing the results between P-spline regression and the Kalman filter, we found that the estimates of the Kalman filter were much more robust to increasing washout times than those from P-spline regression, having a smaller MSE by a factor of 2–10 depending on the metabolic measure. The reason for the bad performance of the P-spline model is that the decomposition is based on the regression of the TEE on the PA, which becomes increasingly more difficult for larger τ since the correlation between TEE and PA fades away. In contrast, the Kalman filter inherently performs deconvolution, which is the preferred approach when τ is large. We therefore also performed P-spline regression on data where the VO_2 and VCO_2 time sequences had been deconvoluted. As shown in **Figure 8**, the results of P-spline regression on deconvoluted data gave much more accurate results, yielding estimation errors that are only a fraction of those that are obtained when increasing sample times or activity noise. For all measures, except the activity RQ and delta activity RQ, the relative MSE is smaller than 0.1% for the whole range of τ using P-spline regression.

It is important to note that in this experiment we modeled the gas diffusion effects of the larger (simulated) chamber as a linear compartment, which was also the main assumption of the deconvolution process. In this idealized situation deconvolution gives extremely accurate results, because the only source of the estimation error is the amplified sensor noise. In practice, therefore, the performance of P-spline regression and Kalman filtering on data measured with a large washout time will be worse because of deviance from the linear compartment model due to non-ideal mixing. Nevertheless, the key point here is that P-spline regression—and any other type of regression approach for that matter—can only work directly on indirect calorimetry data if

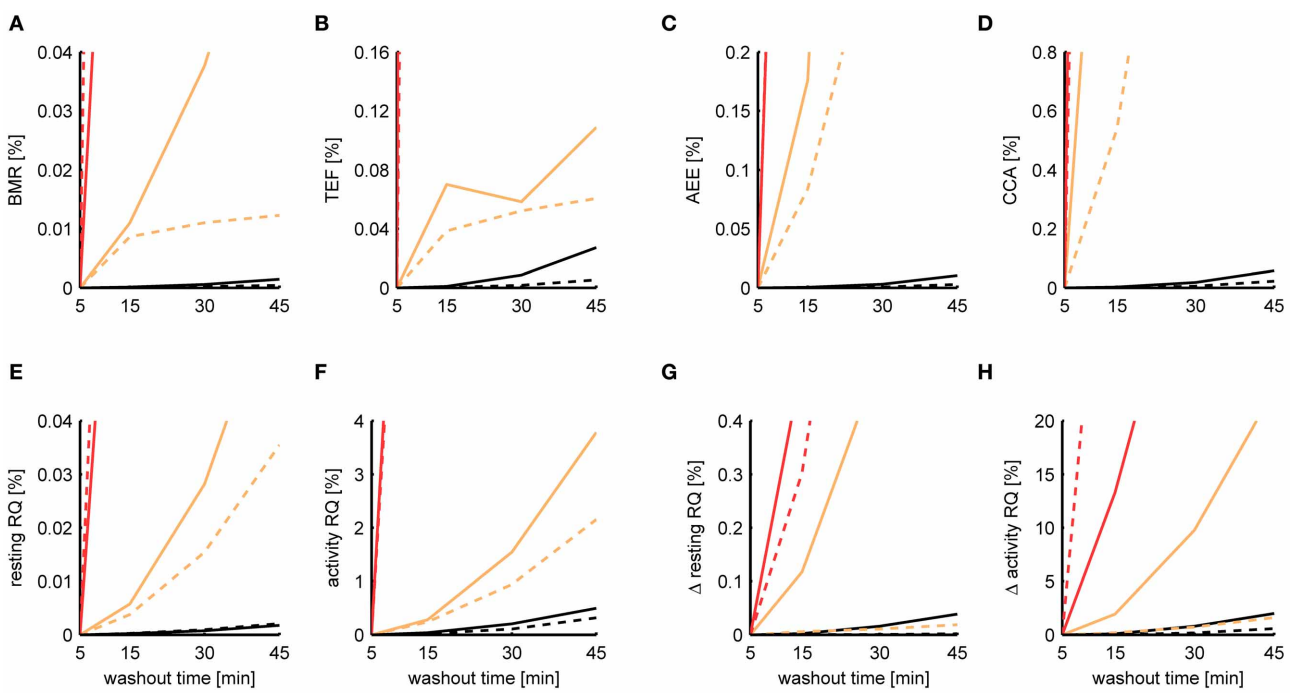


FIGURE 8 | Dependence of the estimation accuracy on the chamber washout time. The relative mean squared error is shown of the BMR **(A)**, TEF **(B)**, AEE **(C)**, CCA **(D)**, resting RQ during fasting **(E)**, activity RQ during fasting **(F)**, increase in resting RQ after feeding **(G)**, increase in activity RQ after feeding **(H)** as estimated by the Kalman filter (orange), P-spline regression (red) and P-spline regression using deconvolution (black) on data from mice (solid) and rats (dashed) with simulated chamber washout times of 5, 15, 30, and 45 min. The relative mean squared error was calculated as the difference of the measure's estimate for a given washout time with the basal estimate at a washout time of 5 min, divided by the basal variance for that measure in mice and rats. Graphs focus on showing the difference between the relative MSE of the Kalman filter and P-spline regression with deconvolution; as a result, the MSE of the ordinary P-spline model lies outside the range of the *y*-axis for most washout times. Note that because part of the Kalman filter's MSE of the TEF estimate is composed of a mean difference, the MSE does not increase monotonically for τ.

the washout time is small, preferably with a chamber volume to air flow ratio of less than 10 min; otherwise the data needs to be deconvoluted first in order to perform robust component analysis.

DISCUSSION

Component analysis has evolved to become an integral part of indirect calorimetry data analysis, and has proved valuable in studies of obesity to elucidate the interaction of energy expenditure with PA (Girardier et al., 1995; Speakman and Selman, 2003; Novak et al., 2006; Kotz et al., 2008; Maclean et al., 2009; Virtue et al., 2012) and food intake (Maclean et al., 2004; Hambly et al., 2005; Johnston et al., 2007). In addition, component analysis has been used to investigate the influence of body composition on the BMR (Johnson et al., 2001; Selman et al., 2001) and of gene mutations (Mokhtarian et al., 1996; Nonogaki et al., 2003). In order to be able to get quantitative insight into these complex interactions and shed light on the mechanisms of body weight regulation, it is essential that the energy components are estimated with maximum accuracy. In this work we discussed the computational techniques that can be used for component analysis and we dealt with issues regarding data preprocessing and the effect of different experimental settings on the estimation accuracy. To fully test the capabilities of these algorithms and compare their results we have used indirect calorimetry data measured in mice and rats.

Making a basal comparison between a set of metabolic measures that were derived from the results of the Kalman filter and P-spline regression, we found that for the BMR, TEF, AEE and resting RQ there was a high agreement between both methods ($R^2 > 0.86$), meaning that for these parameters there is virtually no difference in what method is used. For the CCA the correlation was less strong ($R^2 = 0.59$–0.80), which was mainly caused by the low within group variance for this parameter. In contrast, for the activity RQ in rats large differences were reported between both methods, suggesting that this measure is difficult to estimate reliably, especially if periods of activity are few and short. With the current data it was not possible to determine whether estimates based on the results of either the Kalman filter or P-spline regression were superior. Future research is required to investigate this issue in more depth, for instance by comparing the activity RQ with measures of muscle function.

An important advantage of Kalman filtering and P-spline regression is that they provide time-dependent estimates of the activity and resting VO_2 and VCO_2, which makes it possible to assess the dynamic response of energy metabolism on food intake and other metabolic challenges. Other computational approaches, such as ordinary linear regression and minimal energy expenditure estimation, assume that the RMR is constant and can therefore not be used to look at the time variation in the RMR. These methods are tailored to estimate the RMR on relatively short time intervals when the RMR has stabilized, for instance to determine the BMR.

We compared the BMR estimate obtained by linear regression and the minimal energy expenditure with the estimates from Kalman filtering and P-spline regression, and found that there was a strong correlation between these methods ($R^2 > 0.94$). However, the minimal energy expenditure estimate displayed a downward bias with respect to the other methods, which means that postprocessing of the results of this method is required to correct for this effect. In addition we showed that binning is not as accurate as the linear compartment model to account for the gas mixing effect and to align indirect calorimetry and activity data, because it gives a larger residual error and more uncertainty in the BMR estimate.

Importantly, linear regression in conjunction with binning is not advised for determining the daily AEE and RMR from indirect calorimetry datasets spanning over multiple days, since the resulting estimates will be strongly biased due to the circadian rhythm in the resting energy expenditure. Specifically, the low-pass filter functioning of binning will make it difficult to distinguish between the AEE and the increase in RMR that occurs during the active period of the 24 h cycle, resulting in that the estimate of the daily RMR will approach the RMR of the inactive period of the 24 h cycle, while the AEE will include the increase in RMR during the active period of the 24 h cycle. Therefore, linear regression should always be performed on intervals during which the RMR is stable and by employing a linear compartment model to either convolute the activity data with the impulse response $h(t)$ of the chamber or else deconvolute the calorimetry data.

The ability of the Kalman filter and the P-spline regression model to reliably estimate the time dependency in the resting and activity metabolism comes at the cost of an increased complexity of these methods. Most importantly for the user this means that a number of parameters needs to be set in advance, which affect the performance of the method. For the Kalman filter five parameters need to be set, of which the most important ones are the process noise variances associated with the CCA and RMR. These variances control how quickly each process is allowed to fluctuate and determine whether a change in energy expenditure is attributed to (a change in) the CCA, RMR or measurement noise. The P-spline method has comparable parameters, namely the number of knots that is used in the spline function of the RMR and CCA. An additional parameter that we introduced in this study is the amount of weight that is given in the regression to data measured during activity periods. A weight of zero discards data measured during activity bouts, which has the advantage of decreasing the sensitivity to inaccuracies in the activity data, but it also increases the uncertainty in the global estimate because less data points are used. We found that on the data analysed in this study a relative weight for activity periods of 0.2 gave robust estimates.

It went beyond the scope of the present study to investigate more technical issues regarding the sensitivity of both methods to the choice of their parameters. This is an important direction for future research because some metabolic measures, such as the activity RQ, show a large sensitivity to the parameter choice (**Table 1**). Therefore, more objective criteria need to be found to assist in the standardization of time-dependent component analysis and to eliminate the subjective bias introduced into the estimates of sensitive metabolic measures by the manual tuning of parameters.

The standard for component analysis is to use high time-resolution data, that is, measured with a sample time of 10 s or less, and to use relatively small cages in order to diminish

the effect of gas mixing on the time pattern of the respiratory exchange. Many experimental studies, however, are done in multiplexed systems where respiratory exchange is sampled at a much lower rate and activity is measured with infrared beam breaks. This raises the question of whether any useful metabolic parameters can be derived from the datasets generated by these systems (Even and Nadkarni, 2012). We found in the present study that the BMR and resting RQ during fasting were robust measures against an increasing sample time: for a sample time of 9 min the increase in estimation error in these measures with P-spline regression was not more than 0.3% of the within group variance. The Δ resting RQ after feeding and the TEF and AEE were less robust to changes in the sample time but were still estimated with reasonable accuracy, having a relative error of 3% for a sample time of 9 min. In contrast, the CCA and activity RQ showed a very large sensitivity and could therefore not be reproduced at lower sample rates. Overall the estimation error of the Kalman filter was found to be more sensitive to an increasing sample time than P-spline regression, showing that the latter approach is preferred for analysing data from multiplexed systems.

Based on these data it is difficult to say when the additional estimation error introduced by a lower sample rate has become too large to reliably estimate a given parameter. This will depend on the particular experimental study and, more specifically, on whether the larger number of rodents that can be simultaneously measured in multichannel systems and the fact that animals can typically reside longer in such cages can compensate for the increase in estimation error and the reduction in statistical power. A potential solution to increase the sample rate is to perform continuous data acquisition in multiplexed systems, which permits to predict the actual O_2 and CO_2 concentrations in the present chamber by extrapolating the transitional O_2 and CO_2 concentration of the mixed air by fitting a series of exponential decay curves to the data.

Interestingly, we found that the effect of a reduction in the accuracy of the activity sensor and a larger washout time on the decomposition methods is smaller than the effect of measuring at a low time resolution. In fact, the effect of larger washout times on the estimation accuracy can almost be eliminated, as long as data is measured with a high time-resolution such that deconvolution can be performed. This means that having frequently sampled data is more important for performing robust component analysis than having high accuracy activity sensors or a small washout time.

Concluding, component analysis is an important part of indirect calorimetry data analysis that can provide great additional insight into these datasets. Preferably component analysis is performed on data with a high time-resolution, because this increases the robustness of the decomposition methods, enables the assessment of fast dynamic responses of metabolism on experimental interventions, and permits the calculation of the instantaneous respiratory exchange by means of deconvolution. On low time-resolution data component analysis can be used to measure the BMR or the gradual changes in the RMR associated with circadian rhythm and long term adaptations. The assessment of AEE and TEF can also be feasible in certain cases, but only if the sample time does not exceed 10 min and with the knowledge that the power of subsequent statistical tests may be substantially reduced. On high resolution data from indirect calorimetry systems with continuous data acquisition the Kalman filter and P-spline regression model give very similar results and can therefore both be used. In contrast, for data generated by multichannel system the P-spline regression is advised since it is more robust to infrequently sampled data.

ACKNOWLEDGMENTS

We would like to thank Patrick C. Even for kindly providing the mouse and rat data that has been used in this article. This work was supported by grants from the Netherlands Consortium for Systems Biology (NCSB) and the Center of Medical Systems Biology (CMSB) established by The Netherlands Genomics Initiative/Netherlands Organization for Scientific Research (NGI/NWO). Funding has also been received from TSE systems (Bad Homburg, Germany) in the form of indirect calorimetry equipment. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

Arch, J. R., Hislop, D., Wang, S. J., and Speakman, J. R. (2006). Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals. *Int. J. Obes.* 30, 1322–1331.

Bjursell, M., Gerdin, A.-K., Lelliott, C. J., Egecioglu, E., Elmgren, A., Törnell, J., et al. (2008). Acutely reduced locomotor activity is a major contributor to Western diet-induced obesity in mice. *Am. J. Physiol. Endocrinol. Metab.* 294, 251–260.

Blaxter, K. (1989). *Energy Metabolism in Animals and Man*. Cambridge: Cambridge University Press.

Brown, D., Livesey, G., and Dauncey, M. J. (1991). Influence of mild cold on the components of 24 hour thermogenesis in rats. *J. Physiol.* 441, 137–154.

Bursztein, S., Elwyn, D. H., Askanazi, J., and Kinney, J. M. (1989). *Energy Metabolism, Indirect Calorimetry, and Nutrition*. Baltimore, MD: Williams and Wilkins.

Cannon, B., and Nedergaard, J. (2011). Nonshivering thermogenesis and its adequate measurement in metabolic studies. *J. Exp. Biol.* 15, 242–253.

Cooper, C. E., and Withers, P. C. (2010). Effect of sampling regime on estimation of basal metabolic rate and standard evaporative water loss using flow-through respirometry. *Physiol. Biochem. Zool.* 83, 385–393.

Even, P. C., Mokhtarian, A., and Pele, A. (1994). Practical aspects of indirect calorimetry in laboratory animals. *Neurosci. Biobehav. Rev.* 18, 435–447.

Even, P. C., and Nadkarni, N. A. (2012). Indirect calorimetry in laboratory mice and rats: principles, practical considerations, interpretation and perspectives. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 303, R459–R476.

Even, P. C., and Nicolaidis, S. (1984). Le métabolisme de fond: définition et dispositif de sa mesure. *Comptes Rendus de l'Académie des Sciences* 298, 261–266.

Even, P. C., Perrier, E., Aucouturier, J. L., and Nicolaidis, S. (1991). Utilisation of the method of Kalman filtering for performing the on-line computation of background metabolism in the free-moving, free-feeding rat. *Physiol. Behav.* 49, 177–187.

Feldmann, H. M., Golozoubova, V., Cannon, B., and Nedergaard, J. (2009). UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell Metab.* 9, 203–209.

Girardier, L., Clark, M. G., and Seydoux, J. (1995). Thermogenesis associated with spontaneous

activity: an important component of thermoregulatory needs in rats. *J. Physiol.* 488.3, 779–787.

Hambly, C., Adams, A., Fustin, J. M., Rance, K. A., Bünger, L., and Speakman, J. R. (2005). Mice with low metabolic rates are not susceptible to weight gain when fed a high-fat diet. *Obes. Res.* 13, 556–566.

Heglund, N. C., and Taylor, C. R. (1988). Speed, stride frequency and energy cost per stride: how do they change with body size and gait? *J. Exp. Biol.* 138, 301–318.

Hudson, J. W., and Scott, I. M. (1979). Daily torpor in the laboratory mouse, *Mus musculus* var. albino. *Physiol. Zool.* 52, 205–218.

Humphries, M. M., and Careau, V. (2011). Heat for nothing or activity for free? evidence and implications of activity-thermoregulatory heat substitution. *Integr. Comp. Biol.* 51, 419–431.

Johnson, M. S., Thomson, S. C., and Speakman, J. R. (2001). Limits to sustained energy intake. II. Inter-relationships between resting metabolic rate, life-history traits and morphology in *Mus musculus*. *J. Exp. Biol.* 204.11, 1937–1946.

Johnston, S. L., Souter, D. M., Tolkamp, B. J., Gordon, I. J., Illius, A. W., Kyriazakis, I., et al. (2007). Intake compensates for resting metabolic rate variation in female C57Bl/6J mice fed high-fat diets. *Obesity (Silver Spring)* 15, 600–606.

Kelley, D., and Mandarino, L. (2000). Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes* 49, 677–683.

Kotz, C. M., Teske, J. A., and Billington, C. J. (2008). Neuroregulation of nonexercise activity thermogenesis and obesity resistance. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 294, R699–R710.

Kumahara, H., Schutz, Y., Ayabe, M., Yoshioka, M., Yoshitake, Y., Shindo, M., et al. (2004). The use of uniaxial accelerometry for the assessment of physical-activity-related energy expenditure: a validation study against whole-body indirect calorimetry. *Br. J. Nutr.* 91, 235–243.

Lighton, J. R. B. (2008). *Measuring Metabolic Rates: A Manual for Scientists*. New York, NY: Oxford University Press.

Maclean, P. S., Higgins, J. A., Johnson, G. C., Fleming-Elder, B. K., Donahoo, W. T., Melanson, E. L., et al. (2004). Enhanced metabolic efficiency contributes to weight regain after weight loss in obesity-prone rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 287, R1306–R1315.

Maclean, P. S., Higgins, J. A., Wyatt, H. R., Melanson, E. L., Johnson, G. C., Jackman, M. R., et al. (2009). Regular exercise attenuates the metabolic drive to regain weight after long-term weight loss. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 297, R793–R802.

Meyer, J. A., and Guillot, A. (1986). The energetic cost of various behaviors in the laboratory mouse. *Comp. Biochem. Physiol.* 83, 533–538.

Mokhtarian, A., Decrouy, A., Chinet, A., and Even, P. C. (1996). Components of energy expenditure in the mdx mouse model of Duchenne muscular dystrophy. *Pflugers Arch.* 431, 527–532.

Moran, T. H. (2003). "Methods for the study of the controls of food intake in mice," in *Short Course II: Mouse Behavioral Phenotyping*, ed J. N. Crawley (Washington, DC: Society for Neuroscience), 25–34.

Nonogaki, K., Abdallah, L., Goulding, E. H., Bonasera, S. J., and Tecott, L. H. (2003). Hyperactivity and reduced energy cost of physical activity in serotonin 5-HT(2C) receptor mutant mice. *Diabetes* 52, 315–320.

Novak, C. M., Kotz, C. M., and Levine, J. A. (2006). Central orexin sensitivity, physical activity, and obesity in diet-induced obese and diet-resistant rats. *Am. J. Physiol. Endocrinol. Metab.* 290, E396–E403.

Oppenheim, A. V., Schafer, R. W., and Buck, J. R. (1999). *Discrete-Time Signal Processing*. Upper Saddle River, NJ: Prentice Hall.

Pasquali, V., Scannapieco, E., and Renzi, P. (2006). Validation of a microwave radar system for the monitoring of locomotor activity in mice. *J. Circadian Rhythms* 4, 4–7.

Poirrier, J. E., Poirrier, L., Leprince, P., and Maquet, P. (2006). Gemvid, an open source, modular, automated activity recording system for rats using digital video. *J. Circadian Rhythms* 25, 4–10.

Ravussin, E., Lillioja, S., Anderson, T. E., Christin, L., and Bogardus, C. (1986). Determinants of 24-hour energy expenditure in man – methods and results using a respiratory chamber. *J. Clin. Invest.* 78, 1568–1578.

Reed, G. W., and Hill, J. O. (1996). Measuring the thermic effect of food. *Am. J. Clin. Nutr.* 63, 164–169.

Selman, C., Lumsden, S., Bunger, L., Hill, W. G., and Speakman, J. R. (2001). Resting metabolic rate and morphology in mice (*Mus musculus*) selected for high and low food intake. *J. Exp. Biol.* 204, 777–784.

Speakman, J. R. (2013). Measuring energy metabolism in the mouse - theoretical, practical, and analytical considerations. *Front. Physiol.* 4:34. doi: 10.3389/fphys.2013.00034

Speakman, J. R., Krol, E., and Johnston, M. S. (2004). The functional significance of individual variations in basal metabolic rate. *Physiol. Biochem. Zool.* 77, 900–915.

Speakman, J. R., and Selman, C. (2003). Physical activity and resting metabolic rate. *Proc. Nutr. Soc.* 62, 621–634.

Tokuyama, K., Ogata, H., Katayose, Y., and Satoh, M. (2009). Algorithm for transient response of whole body indirect calorimeter: deconvolution with a regularization parameter. *Appl. Physiol.* 106, 640–650.

Tschöp, M. H., Speakman, J. R., Arch, J. R., Auwerx, J., Brüning, J. C., Chan, L., et al. (2011). A guide to analysis of mouse energy metabolism. *Nat. Methods* 9, 57–63.

Van Klinken, J. B., Van Den Berg, S. A. A., Havekes, L. M., and Willems Van Dijk, K. (2012). Estimation of activity related energy expenditure and resting metabolic rate in freely moving mice from indirect calorimetry data. *PLoS ONE* 7:e36162. doi: 10.1371/journal.pone.0036162

Van Milgen, J., and Noblet, J. (2000). "Modelling energy expenditure in pigs," in *Modelling Nutrient Utilization in Farm Animals*, eds J. P. McNamara, J. France, and D. E. Beever (Oxon: CAB International), 103–114.

Van Milgen, J., Noblet, J., Dubois, S., and Bernier, J.-F. (1997). Dynamic aspects of oxygen consumption and carbon dioxide production in swine. *Br. J. Nutr.* 78, 397–410.

Virtue, S., Even, P. C., and Vidal-Puig, A. (2012). Below thermoneutrality, changes in activity do not drive changes in total daily energy expenditure between groups of mice. *Cell Metab.* 16, 665–671.

Weir, J. B. (1949). New methods for calculating metabolic rate with special reference to protein metabolism. *J. Physiol.* 109, 1–9.

Conflict of Interest Statement: Ko Willems van Dijk has a research collaboration agreement with TSE systems (Bad Homburg, Germany), who also provided funding for the development of the penalised spline regression method in the form of indirect calorimetry equipment. There are no patents or marketed products to declare. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 January 2013; accepted: 12 April 2013; published online: 01 May 2013.

*Citation: van Klinken JB, van den Berg SAA and van Dijk KW (2013) Practical aspects of estimating energy components in rodents. Front. Physiol. **4**:94. doi: 10.3389/fphys.2013.00094*

This article was submitted to Frontiers in Integrative Physiology, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 19 June 2013
doi: 10.3389/fphys.2013.00146

Partitioning of heat production in growing pigs as a tool to improve the determination of efficiency of energy utilization

Etienne Labussière[1,2]*, Serge Dubois[1,2], Jaap van Milgen[1,2] and Jean Noblet[1,2]

[1] *INRA - UMR Pegase, Saint-Gilles, France*
[2] *Agrocampus Ouest - UMR Pegase, Rennes, France*

Edited by:
Patrick C. Even, AgroParisTech, France

Reviewed by:
Cornelia C. Metges, Leibniz Institute for Farm Animal Biology, Germany
Isabelle Ortigues-Marty, Institut National de la Recherche Agronomique, France

****Correspondence:***
Etienne Labussière, INRA - UMR Pegase, F-35590 Saint-Gilles, France
e-mail: etienne.labussiere@rennes.inra.fr

In growing pigs, the feed cost accounts for more than 60% of total production costs. The determination of efficiency of energy utilization through calorimetry measurements is of importance to sustain suitable feeding practice. The objective of this paper is to describe a methodology to correct daily heat production (HP) obtained from measurements in respiration chamber for the difference in energy expenditure related to physical activity between animals. The calculation is based on a preliminary published approach for partitioning HP between HP due to physical activity (AHP), thermic effect of feeding (TEF) and basal metabolic rate (fasting HP; FHP). Measurements with male growing pigs [mean body weight (BW): 115 kg] which were surgically castrated (SC), castrated through immunization against GnRH (IC), or kept as entire male (EM) were used as an example. Animals were fed the same diet *ad-libitum* and were housed individually in two 12-m^3 open-circuit respiration chambers during 6 days when fed *ad-libitum* and one supplementary day when fasted. Physical activity was recorded through interruption of an infrared beam to detect standing and lying positions and with force transducers that recorded the mechanical force the animal exerted on the floor of the cage. Corrected AHP (AHP_c), TEF (TEF_c), and HP (HP_c) were calculated to standardize the level of AHP between animals, assuming that the ratio between AHP_c and ME intake should be constant. Inefficiency of energy utilization (sum of AHP_c and TEF_c) was lower than the inefficiency estimated from the slope of the classical relationship between HP_c and ME intake but was associated with higher requirements for maintenance. Results indicate that EM pigs had higher FHP but lower TEF_c than IC and SC pigs. These results agree with the higher contents in viscera of EM pigs that stimulate their basal metabolic rate and with the reduced utilization of dietary protein to provide energy for maintenance energy requirements and fat deposition (FD).

Keywords: male pig, castrated pig, energy expenditure, physical activity, energy requirements

INTRODUCTION

In growing pigs, feeding accounts for more than 60% of total production costs. The increased use of crop resources for human consumption or fuel production in a context of constrained land resources promotes feedstuff diversification in pig diets, including the use of increasing amounts of by-products (Martin, 2010). Nevertheless, these new feedstuffs are often poorly documented for their energy values, whereas the technological treatments they undergo, often associated with high contents in dietary fiber, may strongly affect metabolic utilization of energy by the growing pigs. Different feeding systems (from digestible energy to net energy, NE) that take into account different energy losses by the animal can be used to describe dietary energy value (Baldwin, 1995a). Among them, the NE system requires measuring energy expenditure associated with the utilization of these feedstuffs for growth (or heat increment HI). The direct measurement in growing animals of heat production (HP) in respiration chamber offers the opportunity to evaluate variation among animals in line with their genotype, phenotype or environmental conditions. Nevertheless, animals produce heat because of different metabolic processes involved in their maintenance and growth functions. The calculation of HI in growing animals needs the partitioning of total HP between a component due to maintenance and a component due to growth. Differences in energy expenditure due to different levels of physical activity between animals have also to be accounted for. The objectives of the paper are to present the methodology developed in our laboratory to calculate HI, using a mathematical model previously described (van Milgen et al., 1997). Further calculations to standardize HI for difference in physical acitivty between animals are proposed. An experiment in which the energy expenditure was measured in entire male (EM) and castrated pigs is used as an example.

MATERIALS AND METHODS

The experiment complied with French laws on animal experimentation and was conducted under the direction of Jean Noblet

and Jaap van Milgen, who are both authorized by the French Ministry of Agriculture (n° 4739 and 7704).

EXPERIMENTAL DESIGN

The experiment was designed to determine the effects of castration and castration method on nitrogen and energy metabolism of male growing pigs. The experiment was conducted on six groups of three Pietrain × (Large White × Landrace) male pigs that were either surgically castrated (SC), immunocastrated (IC), or kept as EM. Within each group, pigs originated from the same litter (five groups) or had the same father (one group) to reduce possible bias in their energy metabolism induced by difference in their genotype. Measurements consisted in 6 days when fed for measuring nitrogen and energy balances (difference between intake and losses in feces, urine and as CH_4 and HP) and 1 day for quantifying fasting HP (FHP) when pigs received no feed. Measurements for IC occurred 5 weeks after the second vaccination when hormonal status of IC pigs was stabilized (Kubale et al., 2013) and measurements for SC and EM pigs occurred simultaneously or 1 week before because only two respiration chambers were available. During measurements, pigs were placed in a metabolic cage allowing quantitative and separate collection of feces and urine and housed individually in a 12-m3 open-circuit respiration chamber, similar as those described by Vermorel et al. (1973). The temperature and relative humidity in the respiration chambers were kept constant at 24°C and 70%, respectively. The pigs were offered a cereal-based diet *ad-libitum* into a trough with a trap door (**Table 1**). A feed hopper placed above the trough ensured that feed was available during the whole day.

Table 1 | Composition of diet.

INGREDIENTS (%)	
Corn	16.00
Wheat	26.20
Barley	25.55
Soybean meal	19.00
Vegetable fat	2.00
Molasses	3.00
Wheat bran	5.00
Bicalcium phosphate	0.50
Calcium carbonate	1.29
Sodium chloride	0.45
L-lysine-HCl	0.33
DL-methionine	0.04
L-threonine	0.03
Vitamins, oligoelements and phytase	0.61
CHEMICAL COMPOSITION (% OF DM)	
Crude protein	20.11
Starch	44.66
Crude fat	4.0
Crude fiber	4.0
NDF	17.0
ADF	4.9
ADL	0.9
Gross energy (MJ/kg DM)	18.61

MEASUREMENTS AND SAMPLINGS

Pigs were weighed on the morning of the first day of measurements, on the morning of the fasting day and on the morning after the fasting day. The amount of feed offered was recorded daily and feed refusals and spillages were collected at the end of the 6 fed days. Offered feed was sampled daily for each week of measurements. At the end of each week, feces from each pig were weighed, mixed, and sampled. Urine was weighed daily and a daily aliquot was cumulated over the 6 days of the fed period for each pig. Ammonia losses that resulted from the degradation of urinary nitrogen were recovered from the condensed water from the air conditioning system while ammonia losses in outgoing air were determined as described by Noblet et al. (1987).

According to the open-circuit respiration chamber technique, volumes of O_2 consumption and CO_2 and CH_4 production were calculated from ventilation rate of the respiration chamber and from the difference in gas concentrations between outgoing and ingoing air. The O_2, CO_2, and CH_4 concentrations in outgoing air were measured using a paramagnetic differential analyzer (Oxymat 6, Siemens) and two infrared analyzers (Ultramat 6, Siemens), respectively. The ventilation rate was measured with a mass gas meter (Teledyne Brown Engineering). Gas concentrations, ventilation rate and physical characteristics of the gas in the respiration chamber (pressure, temperature, and relative humidity) were measured 60 times per second, averaged over 10-s intervals and recorded for further calculations. Each day, access to the feeder was blocked at 6.00 am and measurements were stopped at 8.00 am for ∼15 min to provide care to the animals, refill the feeders, collect feces and urine and calibrate the analyzers with ingoing air as baseline and a gas tube with known gas concentrations as standard. Measurements then restarted and access to feeder was allowed at 9.00 am.

Feeding behavior was recorded continuously using a weighing scale that was placed under the trough. Standing duration was recorded through interruption of an infra-red beam that was placed across the cage at the height of the pig's hip. The mechanical force the pig exerted because of physical activity was recorded using four force sensors (9104A, Kistler) on which the cage was placed. The sensors are transducers that produce an electrical signal proportional to the vertical force the animal exerts on the cage (Quiniou et al., 2001).

LABORATORY ANALYSES

Feed samples and feed refusals were analyzed weekly for dry matter (DM) content. Feed samples were then pooled and analyzed for DM, nitrogen (Dumas method) and energy contents (AOAC, 1990; AFNOR, 1998). One sample of feces per pig was analyzed for DM content and one sample was freeze-dried. Freeze-dried feces samples were ground through a 1 mm grid and analyzed for DM, nitrogen (Dumas method) and energy contents. Nitrogen content of urine was measured on fresh material according to the Dumas method and energy content was measured after freeze-drying approximately 30 mL of urine in polyethylene bags (AFNOR, 1998).

CALCULATIONS

Gas analyzers were calibrated at the beginning and at the end of each day and the drift was considered to be linear. The time lag between respiration chamber and gas analyzers equaled 70 s. Taking into account the effect of respiratory quotient (RQ, CO_2/O_2) on difference between inflow and outflow (Ortigues et al., 1994), volumes of O_2 consumption and CO_2 and CH_4 production were calculated for 10-s intervals that were cumulated over the day. To account for the interruption of the measurements in the morning (calibration of analyzers . . .), these volumes were standardized for 24-h period, assuming proportionality.

Nitrogen balance was calculated as the difference between intake (calculated as the difference between offered feed and feed refusals and spillages) and losses in feces and urine and as ammonia. Protein deposition (PD) was then calculated, assuming that PD equaled 6.25 times nitrogen retention. Retained energy (RE) was calculated as the difference between feed gross energy intake and energy losses in feces and urine and as CH_4 (39.5 kJ/L of CH_4) and HP. According to the Brouwer (1965) equation, HP was calculated from volumes of O_2 consumption, CO_2 production, and CH_4 production and nitrogen excreted in urine (including ammonia losses). Fat deposition (FD) was calculated from the energy balance, assuming that energy was retained only as protein (23.6 kJ/g PD) and as fat (39.7 kJ/g FD).

MATHEMATICAL MODELING OF HP PARTITION

Total HP was partitioned between components due to basal metabolism FHP, physical activity (AHP) and thermic effect of feeding (TEF, **Figure 1**) through analysis of the dynamic patterns of O_2 and CO_2 concentrations in the air of the respiration chamber (van Milgen et al., 1997). The model assumes that the instantaneous variations in O_2 and CO_2 concentrations are related to O_2 consumption and CO_2 production by the pig (sub-model "animal"; **Figure 2**), in addition to variation induced by ventilation of the respiration chamber and variation of physical characteristics of the gas within the respiration chamber (sub-model "chamber"). A complete description of the mathematical model is given by van Milgen et al. (1997).

Mathematical modeling of gas exchanges

The conception of the model was similar for O_2 consumption and CO_2 production. During the fed days, the sub-model "animal" considered that instantaneous O_2 consumption or CO_2 production (in standard conditions of temperature and pressure: 0°C and 1 atm) equaled the sum of O_2 consumption or CO_2 production due to physical activity and short-term TEF (TEF_s), in addition to constant O_2 consumption or CO_2 production associated with resting metabolism (VOFED and VCFED, respectively; **Figure 2**). It was hypothesized that O_2 consumption or CO_2 production due to physical activity was proportional to the electrical signal from force sensors with different parameters for O_2 and CO_2 (VOFOR and VCFOR, respectively). The O_2 consumption or CO_2 production due to TEF_s followed a gamma distribution. The latter was modeled as the output of a two-compartment system, which was filled in the first compartment by feed intake (recorded by the weighing scale placed under the trough) and parameterized by the volume of O_2 consumed or CO_2 produced per g of feed intake (O2TEF and CO2TEF, respectively) and by mean time between feed intake and its related O_2 consumption or CO_2 production (TTEF). Mathematically, the content

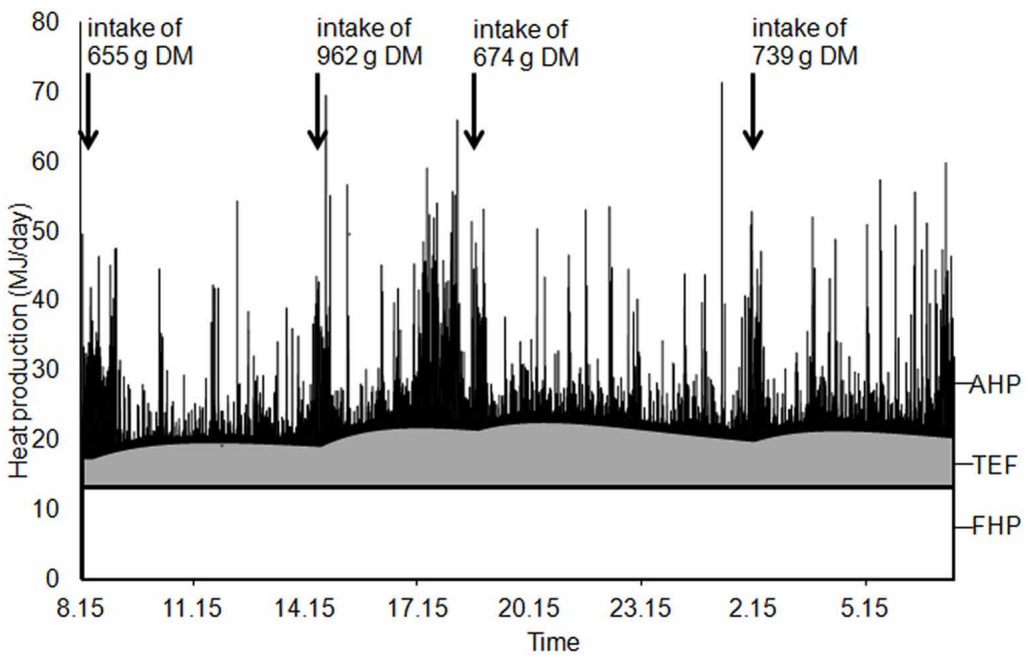
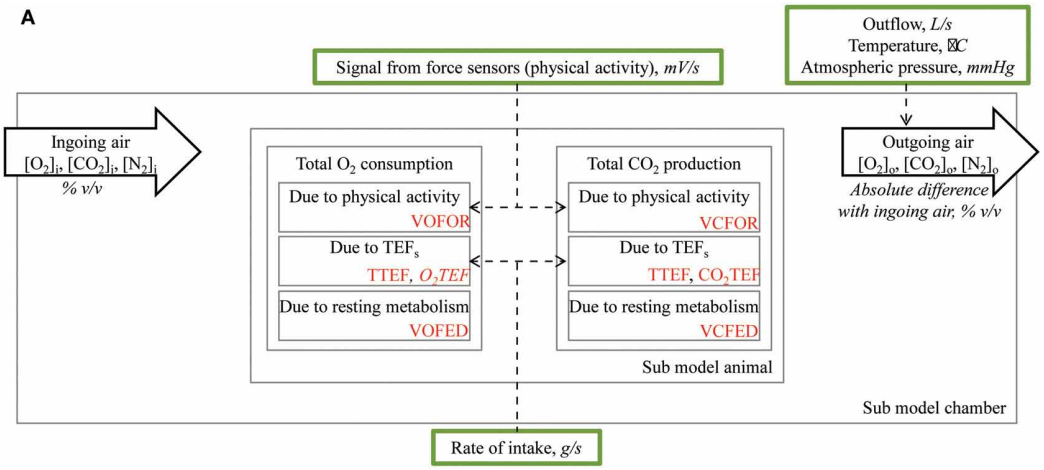


FIGURE 1 | Example of heat production partitioning between components due to basal metabolic rate (fasting heat production, FHP), physical activity (AHP) and thermic effect of feeding (TEF); IC pig from group 3.



Parameters of the model:

VOFED (VCFED): O_2 consumption (CO_2 production) due to resting metabolism when fed, *L/h*

O_2TEF (CO_2TEF): O_2 consumption (CO_2 production) due to feed intake, *L/g of feed*

TTEF: mean time between feed intake and its related O_2 consumption and CO_2 production, *h*

VOFOR (VCFOR): O_2 consumption (CO_2 production) due to physical activity, *L/mV from force sensors*

$[O_2]_i$: O_2 concentration in ingoing air, *20.95 % v/v*

$[CO_2]_i$: CO_2 concentration in ingoing air, *0.03 % v/v*

$[N_2]_i$: N_2 concentration in ingoing air, *79.02 % v/v*

Outputs of the model:

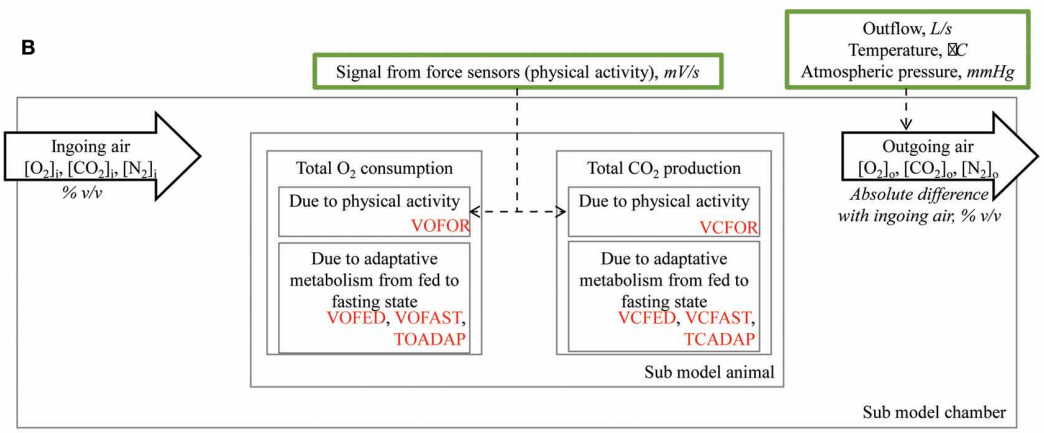
$[O_2]_o$: O_2 concentration in outgoing air, % v/v

$[CO_2]_o$: CO_2 concentration in outgoing air, % v/v

$[N_2]_o$: N_2 concentration in outgoing air, % v/v

Inputs of the model are in green boxes.

Units are indicated in italic.



Parameters of the model:

VOFED (VCFED): O_2 consumption (CO_2 production) due to resting metabolism when fed, *L/h*

VOFAST (VCFAST): O_2 consumption (CO_2 production) due to basal metabolic rate, *L/h*

TOADAP (TCADAP): rate of decline of O_2 consumption (CO_2 production) due to adaptative metabolism from fed to fasted state, */h*

VOFOR (VCFOR): O_2 consumption (CO_2 production) due to physical activity, *L/mV from force sensors*

$[O_2]_i$: O_2 concentration in ingoing air, *20.95 % v/v*

$[CO_2]_i$: CO_2 concentration in ingoing air, *0.03 % v/v*

$[N_2]_i$: N_2 concentration in ingoing air, *79.02 % v/v*

Outputs of the model:

$[O_2]_o$: O_2 concentration in outgoing air, % v/v

$[CO_2]_o$: CO_2 concentration in outgoing air, % v/v

$[N_2]_o$: N_2 concentration in outgoing air, % v/v

Inputs of the model are in green boxes.

Units are indicated in italic.

FIGURE 2 | Description of the mathematical model used to partition total heat production from kinetics of O_2 consumption and CO_2 production; (A) components when animals are in a fed state; **(B)** components when animals are in a fasted state.

of each compartment was modeled from its first-order derivative with respect to time and fractional emptying rates were assumed to be identical for both compartments (2/TTEF; van Milgen et al., 1997). In addition to these well-identified contributors to O_2 consumption and CO_2 production, early experiments indicated that modest and time-limited variations in O_2 and CO_2 concentrations in the respiration chamber can occur irrespective of feed intake or physical activity (van Milgen and Noblet, 2000). Although the contribution of these phenomena to the total volumes of O_2 consumption or CO_2 production is small (<0.5%), they can affect the estimates of parameters of the model when not accounted for. These events were manually included in the model to ensure proper parameter estimation as instantaneous O_2 consumption and CO_2 production. During the fasting day, there is no feed intake. However, O_2 consumption or CO_2 production during resting (when the contribution of physical activity was removed) are lower during fasting than when fed. The decline in O_2 consumption or CO_2 production was described as a first-order decline between O_2 consumption or CO_2 production at a fed state (VOFED and VCFED, respectively) and O_2 consumption or CO_2 production during fasting (VOFAST and VCFAST, respectively). It was hypothesized that the rate of decline (TOADAP and TCADAP, respectively) may be different for O_2 and CO_2. Finally, the sub-model "animal" allowed calculating O_2 consumption and CO_2 production using feed intake and signals from the force sensors as inputs and seven parameters for the fed days and eight parameters for the fasting day.

The sub-model "chamber" described the variation in physical characteristics of the gas and considered that the air in the respiration chamber was composed of O_2, CO_2, and N_2. Because only the flow of outgoing air was measured, the inflow was calculated as the flow of air required to fill the physical volume of the respiration chamber when O_2 consumption, CO_2 production and outflow were considered; the physical volume of the respiration chamber was calculated in standard conditions of temperature and pressure (0°C, 1 atm). The concentration of each gas in the respiration chamber was then calculated from its volume divided by the sum of volumes of O_2, CO_2, and N_2.

Equations of the model were written in Fortran and compiled in a dynamic linked library that was loaded in R (R Development Core Team, 2010). Package deSolve (Soetaert et al., 2010) was used to solve the ordinary differential equations with an integration step-size of 10 s, after smoothing the data from force sensors, temperature, pressure and outflow to ensure their continuity. Parameters of the model were estimated for each day according to a three-step procedure: parameters directly related to O_2 consumption or CO_2 production were first estimated separately and then together to minimize the sum of squared differences between predicted O_2 or CO_2 concentration and measured O_2 or CO_2 concentrations (Nelder and Mead, 1965).

Calculation of HP components

Energy expenditure due to fasting metabolism, physical activity and TEF_s were calculated from the respective volumes of O_2 consumption and CO_2 production according to the Brouwer (1965) equation. The difference between resting HP when fed (i.e., total HP minus AHP and TEF_s) and FHP was attributed to long-term TEF (TEF_l) and total TEF was calculated as the sum of short- and long-term components.

Standardization of HP for differences in physical activity

Preliminary analysis on data indicated that correlation between AHP and ME intake was significant ($r = 0.56$, $P < 0.05$; **Table 2**). To standardize HP between animals for difference in their physical activity, it was assumed that a fixed amount of metabolizable energy (ME) intake should be dissipated as corrected AHP (AHP_c). The proportion of ME intake that was dissipated as HP due to physical activity equaled the mean value of AHP/ME (8.6%; see results). When AHP was higher than AHP_c, the difference between AHP and AHP_c resulted in a positive variation of ME available for other metabolic pathways, which was dissipated as TEF or retained as fat. The amount which was dissipated as supplementary TEF was calculated as: (AHP—AHP_c) × TEF / (ME—FHP—AHP) and was added to TEF to calculate a corrected TEF (TEF_c). The difference between AHP and AHP_c which was not dissipated as supplementary TEF was added to RE to calculate corrected RE (RE_c) and FD (FD_c). When AHP was lower than AHP_c, the standardization followed the same calculation routine but resulted in lower TEF_c, RE_c and FD_c than TEF, RE, and FD, respectively. Assuming that FHP is representative of the basal metabolic rate of *ad-libitum* fed animals (Baker et al., 1991), HI was calculated as the sum of AHP_c and TEF_c. The efficiency of utilizing ME for maintenance and growth (k_{mg}, %) was calculated as (1-HI/ME) × 100. Maintenance ME requirements (ME_m) were calculated as FHP × $100/k_{mg}$ (Labussière et al., 2011). All energy traits were expressed relative to metabolic body size, which was calculated as body weight (BW) raised to the power 0.60 (Noblet et al., 1999).

The NE intake was calculated as the difference between ME intake and HI. The energy values of the diet (ME and NE contents) were calculated as the ratio between ME or NE intake (MJ/day) and feed intake.

STATISTICAL ANALYSES

One FHP value was missing for a SC pig in group 2 and it was calculated (kJ/kg $BW^{0.60}$ per day) as the average of the values

Table 2 | Pearson correlation coefficients between time spent standing (h/d), mean voltage measured from force sensors (mV/d), ME intake (kJ/kg $BW^{0.60}$ per day) and physical activity heat production (AHP; kJ/kg $BW^{0.60}$ per day) and their ratio (AHP/ME; %).

	Mean voltage from force sensors	ME intake	AHP	AHP/ME
Time spent standing	0.12	0.19	0.03	−0.09
Mean voltage from force sensors		0.65**	0.85**	0.52*
ME intake			0.56*	−0.09
AHP				0.77**

*$P < 0.05$; **$P < 0.01$.

obtained for the SC in the five other groups. The data ($n = 18$) were analyzed for the effects of sex (EM, SC, IC) and group using the PROC GLM of SAS (SAS, 2004). Only the *P*-values for the effect of sex will be described in details. Pearson correlation coefficients between time spent standing, mean voltage measured from force sensors, ME intake, AHP and AHP/ME ratio were calculated (PROC CORR; SAS, 2004). The linear relationship between AHP (% of ME intake) and the mean voltage from force sensors (mV/day) was tested and the difference of the slope from zero was tested through a *T*-test (PROC GLM; SAS, 2004). The linear relationship between corrected HP_c (HP_c) (kJ/kg $BW^{0.60}$ per day) and ME intake (kJ/kg $BW^{0.60}$ per day) was tested for the effect of sex on intercept and slope of the relationship (PROC GLM; SAS, 2004).

RESULTS

METHODOLOGICAL ASPECTS

The BW of the pigs did not differ between sexes and averaged 115 kg during measurements (**Table 3**). Voluntary ME intake varied significantly between 2396 kJ/kg $BW^{0.60}$ per day for EM pigs to 2864 kJ/kg $BW^{0.60}$ per day for IC pigs. There was no effect of sex on time spent standing that averaged 1.4 h/day but individual values varied from 0.9 to 2.0 h/day (**Figure 3**). The force the animals exerted on the floor (mean voltage measured from force sensors) varied from 1.6 to 4.0 mV/day (**Figure 4**) and it was significantly correlated with ME intake, AHP and AHP/ME intake (**Table 2**). The AHP did not differ significantly between sexes (**Table 3**) but it was significantly correlated with ME intake (**Table 2**). When expressed as a percentage of ME intake, AHP was not affected by sex and averaged 8.6% (**Table 3**). Additionally, it was significantly correlated with mean voltage from force sensors (**Table 2**).

ENERGY BALANCE

Total HP_c tended to vary according to the same pattern as ME intake from 1376 to 1519 kJ/kg $BW^{0.60}$ per day ($P = 0.06$; **Table 3**). The relationship between HP_c and ME intake did not differ significantly between sexes; the intercept equaled 554 kJ/kg $BW^{0.60}$ per day and the slope equaled 34%. Among HP_c components, FHP was significantly higher for EM pigs (856 vs. 761 kJ/kg $BW^{0.60}$ per day on average for castrated pigs) whereas TEF_c was significantly lower for EM pigs (315 vs. 474 kJ/kg $BW^{0.60}$ per day or 13.0 vs. 17.2% of ME intake on average for castrated pigs). When HP due to physical activity was corrected for the differences between animals, HI was significantly lower in EM pigs than in castrated pigs (522 vs. 712 kJ/kg $BW^{0.60}$ per day, on average). Variations in ME intake and energy expenditure resulted in lower RE_c in EM than in castrated pigs (1020 vs. 2562 kJ/kg $BW^{0.60}$ per day, on average). Additionally, inefficiency of utilizing ME for maintenance and growth (i.e., HI_c expressed as % of ME intake) was significantly lower in EM than in castrated pigs (21.6 vs. 25.9%, on average for castrated pigs). Maintenance ME requirements varied among pigs and ranged from 997 for SC pigs to 1091 kJ/kg $BW^{0.60}$ per day for EM pigs. The RQ was significantly lower in EM pigs than in castrated pigs, irrespective of castration method (1.08 vs. 1.15). Dietary ME content tended to vary between 15.13 in IC pigs to 15.41 MJ/kg DM in EM pigs. The NE content of the diet was significantly higher for EM pigs (12.02 vs. 11.34 MJ/kg DM on average for castrated pigs).

Table 3 | Effect of castration and castration method on energy balance, efficiency of utilizing ME for maintenance and growth and maintenance energy requirements in male growing pigs (results are LS-means; $n = 18$).

	Sex				
	EM	**SC**	**IC**	**Rsd**	***P*-value**
BW (kg)	114.0	111.0	120.1	7.5	0.15
Time spent standing (h/day)	1.6	1.3	1.3	0.4	0.42
ME intake (kJ/kg $BW^{0.60}$ per day)	2396[b]	2632[a,b]	2864[a]	208	<0.01
ENERGY EXPENDITURE (kJ/kg $BW^{0.60}$ PER DAY)					
FHP	856[a]	735[b]	783[b]	36	<0.01
AHP	218	212	250	38	0.22
AHP_c	207[b]	227[a,b]	247[a]	18	<0.01
TEF_c	315[b]	464[a]	484[a]	65	<0.01
HI	522[b]	692[a]	732[a]	81	<0.01
HP_c	1376	1416	1519	91	0.06
RE_c (kJ/kg $BW^{0.60}$ per day)	1020[b]	1216[a]	1346[a]	125	<0.01
ENERGY EXPENDITURE (% of ME INTAKE)					
AHP	9.2	8.0	8.7	1.5	0.45
AHP_c	8.6	8.6	8.6	–	
TEF_c	13.0[b]	17.5[a]	16.9[a]	1.6	<0.01
HI_c	21.6[a]	26.2[b]	25.6[b]	1.6	<0.01
ME_m (kJ/kg $BW^{0.60}$ per day)	1091[a]	997[b]	1054[a,b]	42	0.02
Respiratory quotient	1.08[b]	1.14[a]	1.15[a]	0.03	<0.01
DIETARY ENERGY VALUE (MJ/kg DM)					
ME	15.41	15.37	15.13	0.19	0.06
NE	12.02[a]	11.42[b]	11.25[b]	0.35	<0.01

EM, entire male pigs; SC, surgically castrated pig; IC, immuno-castrated pig; Rsd, residual standard deviation; BW, body weight; ME, metabolizable energy; FHP, fasting heat production; AHP, physical activity related heat production; TEF_c, thermic effect of feeding corrected for inter-individual differences in energy expenditure due to physical activity; HI, heat increment; HP_c, total heat production corrected for inter-individual differences in energy expenditure due to physical activity; RE_c, retained energy corrected for inter-individual differences in energy expenditure due to physical activity; ME_m, maintenance metabolizable energy requirements; NE, net energy; Measurements started 5 weeks after the second vaccination for IC pigs. Only 5 data for SC pigs were available for calculating FHP.

[a, b] Within the same row; LS-means with different superscripts differ ($P < 0.05$).

NUTRIENT DEPOSITION

The BW gain was calculated from BW measured at the beginning and at the end of the 6 days of balance measurement; it did not differ between sexes and averaged 1273 g/day (**Table 4**). From balance measurements, PD was significantly lower for SC pigs (196 vs. 254 g/day on average for EM and IC pigs) whereas FD_c was significantly lower for EM pigs (288 vs. 429 g/day on average for SC

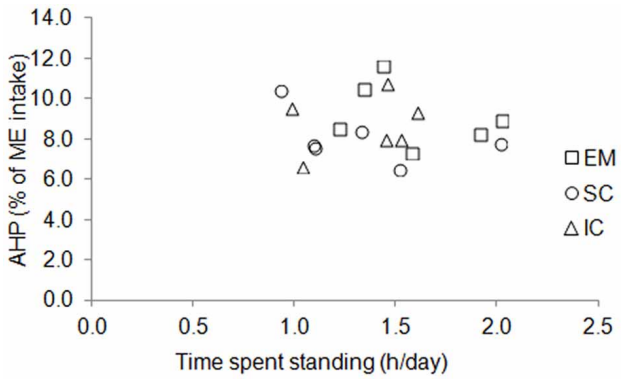


FIGURE 3 | Individual variations of time spent standing and energy expenditure due to physical activity (AHP, % of ME intake) in entire male (EM), surgically castrated (SC), and immune-castrated (IC) pigs.

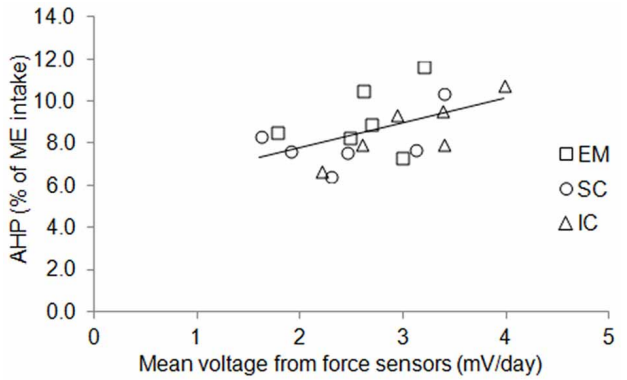


FIGURE 4 | Individual variations of mean voltage measured from force sensors and energy expenditure due to physical activity (AHP, % of ME intake) in entire male (EM), surgically castrated (SC) and immune-castrated (IC) pigs. Solid line: linear relationship between AHP (% of ME intake) and cumulative voltage from force sensors (mV/day); the slope equaled 1.2% of ME per mV and differed significantly from zero ($P = 0.03$).

and IC pigs). Accordingly, the FD_c content of BW gain was lower for EM pigs whereas the PD content of BW gain did not differ significantly between sexes and averaged 234 g/day.

DISCUSSION

METHODOLOGICAL ASPECTS FOR MEASURING NET ENERGY VALUE OF A DIET

The evaluation of the energy value of feedstuffs and feeds requires estimating the efficiency of energy utilization of nutrient utilization by animals. In growing animals, theoretical calculations involve the artificial distinction between energy use for maintenance and for growth and require several assumptions regarding metabolic pathways and composition of BW gain (protein and lipid deposition, protein and lipid turnover, fatty acid composition of *de novo* lipid synthesis). Additionally, these calculations do not account for the energy costs associated with ingestion and digestion of feed. Alternatively, calorimetry measurements

Table 4 | Effect of castration and castration method on BW gain and its composition in male growing pigs (results are LS-means; $n = 18$).

	Sex				
	EM	**SC**	**IC**	**Rsd**	***P*-value**
BW gain (kg/day)	1370	1133	1317	288	0.37
NUTRIENT DEPOSITION (g/day)					
PD	261[a]	196[b]	246[a]	28	<0.01
FD_c	288[b]	403[a]	454[a]	51	<0.01
BODY GAIN COMPOSITION (g/kg of BW GAIN)					
PD	261	196	246	43	0.53
FD_c	284[b]	407[a]	453[a]	41	<0.01

EM, entire male pigs; SC, surgically castrated pig; IC, immune-castrated pig; Rsd, residual standard deviation; BW, body weight; PD, protein deposition; FD_c, fat deposition corrected for inter-individual differences in energy expenditure due to physical activity; Measurements started 5 weeks after the second vaccination for IC pigs.

[a, b] Within the same row; LS-means with different superscripts differ ($P < 0.05$).

in living animals allow estimating an overall efficiency of utilizing dietary energy for maintenance and growth and they include the associated energy costs. In this paper, efficiency was calculated from the inefficiency due to TEF and AHP. Nevertheless, it could be biased by differences in physical activity among animals (i.e., social confinement, reduced physical activity because of contention).

Several methods have been used in the past to quantify physical activity and to link physical activity to energy expenditure. In pigs, these methods have been based on regression analyses between HP and time budget that was determined using infrared barriers (e.g., Noblet et al., 1993) or video recordings (e.g., Rijnen et al., 2003) but these methods do not allow quantifying the level of physical activity (i.e., the mechanical force and the associated efficiency the animal develops because of its physical activity). In this way, results from our experiment indicate that the time the animals spent standing has little effect on AHP (**Figure 3**). Indeed, the time the pig was standing was measured through an infra-red barrier, which was placed across the cage at the height of the pig's hip. Consequently, standing also included other activities like sitting, rubbing, walking (only to small extent because of the cage), or digging. The quantification of physical activity requires measuring traits which are thought to be proportional to the mechanical force which is exerted by the animal. Indeed, ultrasonic burglars were used in pigs (e.g., Schrama et al., 1998) and more recently, accelerometers have been proposed to measure physical activity in rodents and humans. Nevertheless, these measurements may be subject to errors in estimating accurately physical activity of large animals because measured values can be specific to a given physical activity. The consequence is that measured values can be less representative of the physical activity of the whole body, depending on the position of the ultrasonographic burglar devices relative to the body of the animal, or the position of the accelerometer on the body of the animal. In our experiments and in others (e.g., Even et al., 1991), the cage where the animals were housed was located on force transducers that are sensors that produce

an electrical signal proportional to the force the animal exerts on the floor. The partitioning of total HP to determine what is due to physical activity then requires estimating the amount of energy expenditure per unit of electrical signal from force sensors and involves parameter optimization through mathematical modeling. Using the signals from force transducers, the latter has been performed through Kalman filtering (Kalman, 1960; Even et al., 1991) or Nelder–Mead minimization (Nelder and Mead, 1965; van Milgen et al., 1997). In our approach, parameter optimization includes also the determination of energy cost associated with TEF and resting metabolism. In this paper, the determination coefficient of the variations in gas concentrations by the mathematical model averaged 92% over the 126 days that were modeled (18 pigs with 7 days each). Nevertheless, the model considers that each unit of electrical signal from force transducers corresponds to a fixed volume of consumed O_2 and produced CO_2 and does not account for the metabolic difference in muscles involved in physical activity between standing and lying.

EFFICIENCY OF UTILIZING ENERGY IN GROWING PIGS

Growing animals produce heat because of their maintenance and growing metabolism. Classically, the slope of the relationship between HP_c and ME intake (34% in our experiment) was considered as an estimate of the inefficiency of utilizing dietary energy but this approach has been questioned because of the adaptation of animal to feeding level (de Lange et al., 2006; Labussière et al., 2011). In the modelling approach for partitioning HP, AHP and TEF are indicative for the inefficiency in utilizing dietary energy whereas FHP is indicative of the basal metabolic rate of animals (Labussière et al., 2011). This inefficiency varied from 22% in EMs to 26% in castrated pigs which agrees with previous results (Labussière et al., 2011). These values were also lower than those estimated from the classical regression between HP_c and ME intake but they were associated with higher values of maintenance energy requirements (Labussière et al., 2011).

Irrespective of castration method, AHP accounted for 8.6% of ME intake, which agrees with previous observations in growing pigs fed close to *ad-libitum* (from 7.6 to 11.6% of ME intake; Schrama et al., 1998; Le Bellego et al., 2001; Quiniou et al., 2001; van den Borne et al., 2007; Labussière et al., 2011; Renaudeau et al., 2013) but values were highly variable between animals (**Figure 3**). To account for the possible bias induced by the variation in AHP between animals, a calculation routine was used to standardize AHP between animals, which resulted in variations in TEF_c and RE_c. In our experiment, TEF_c was higher in SC and IC pigs than in EM (**Table 3**). Values for SC or IC pigs agree with previous results in SC pigs which received a similar diet (16.8% of ME intake; Barea et al., 2010). Data for TEF_c in EM pigs are scarce but the differences in TEF_c between EMs and castrated pigs agree with the differences in metabolism of nutrients due to lower feed intake, higher PD and lower lipid deposition that result in a lower RQ in EM pigs. Indeed, theoretical calculations for energy efficiencies for lipid deposition are always lower when the energy is provided by proteins rather than by carbohydrates or lipids (Armstrong, 1969). Calculations using diet composition and the difference between digested N and N deposited in PD (**Table 4**) indicate that dietary protein contributed to 13% in EM and 18% in SC of the energy used for maintenance and lipid deposition, which agrees with the lower TEF_c in EM pigs. Consequently, dietary NE content, which is thought to be representative of the true energy value of the diet, depended on the sexual type of the animal and it was higher in EM pigs (**Table 3**).

MAINTENANCE ENERGY METABOLISM IN GROWING PIGS

During the fasting day, the mathematical modeling of HP was considered to occur according a first-order decrease in energy metabolism between fed and fasted states. The FHP was calculated as the asymptotic value of resting HP (van Milgen et al., 1997). These values of FHP exclude the energy expenditure due to physical activity and the values for SC pigs agree with values previously estimated using a similar methodology (711 to 846 kJ/kg $BW^{0.60}$ per day; Le Bellego et al., 2001; Le Goff et al., 2002; Lovatto et al., 2006; Barea et al., 2010; Labussière et al., 2011). According to previous results with growing pigs (van Milgen et al., 1998), FHP of EM pigs was higher than that of castrated pigs (**Table 3**). This result agrees with the greater mass of viscera in EM than in SC (Quiniou and Noblet, 1995), which influences FHP (Koong et al., 1982, 1985; Pekas and Wray, 1991) because of the greater energy requirements of the portal-drained viscera (Johnson et al., 1990; Ortigues et al., 1995). Estimating FHP allows determining ME_m in growing animals as the ratio between FHP and k_{mg} (Labussière et al., 2009) without involving the classical regression analyses between RE and ME intake (Kielanowski, 1965; Baldwin, 1995b). The classical regression analysis has been criticized because of the adaptation of the animal to feeding level (de Lange et al., 2006; Labussière et al., 2011). In our experiment, ME_m was higher in EM than in SC pigs, which disagrees with previous results (Noblet et al., 1999). Nevertheless, the latter values were calculated from the classical regression methods and were obtained with pigs at lower BW (i.e., younger) than those in the present study. The difference in energy metabolism between entire and castrated males may be less pronounced because of less advanced sexual maturity.

In conclusion, mathematical modeling of daily dynamics of HP and accounting for the variation in physical activity among animals allows calculating the energy expenditure due to physical activity and TEF, in addition to the HP due to basal metabolic rate. In growing animals, the energy utilization of the diet depends on metabolic pathways involved in maintenance and lipid deposition, according to the nutrients that are used. Consequently, the dietary NE content depends on the sexual type of growing animals.

ACKNOWLEDGMENTS

The authors would like to thank E. Gonzalo, R. Janvier, Y. Jaguelin-Peyaud, A. Pasquier and V. Piedvache for animal care and laboratory analyses.

REFERENCES

AFNOR. (1998). Norme ISO 9831:1998. *Détermination de la Valeur Calorifique Brute. Méthode à la Bombe Calorimétrique*. Genève: Organisation Internationale de Normalisation.

AOAC. (1990). *Official Methods of Analysis*. Arlington, TX: Association of Official Analytical Chemists.

Armstrong, D. G. (1969). "Cell bioenergetics and energy metabolism," in *Handbuch der Tierernährung*, eds W. Lenkeit, K. Breirem, and E. Crasemann (Hamburg: Verlag P. Parey), 385–414.

Baker, J. F., Buckley, B. A., Dickerson, G. E., and Nienaber, J. A. (1991). Body composition and fasting heat production from birth to 14 months of age for three biological types of beef heifers. *J. Anim. Sci.* 69, 4406–4418.

Baldwin, R. L. (1995a). "Animal energetic models," in *Modeling Ruminant Digestion and Metabolism*, ed R. L. Baldwin (London: Chapman and Hall), 118–147.

Baldwin, R. L. (1995b). "Energy requirements for maintenance and production," in *Modeling Ruminant Digestion and Metabolism*, ed R. L. Baldwin (London: Chapman and Hall), 148–188.

Barea, R., Dubois, S., Gilbert, H., Sellier, P., van Milgen, J., and Noblet, J. (2010). Energy utilization in pigs selected for high and low residual feed intake. *J. Anim. Sci.* 88, 2062–2072. doi: 10.2527/jas.2009-2395

Brouwer, E. (1965). "Report of subcomittee on constants and factors," in *Energy Metabolism*, ed K. L Blaxter (London: Academic Press), 441–443.

de Lange, K., van Milgen, J., Noblet, J., Dubois, S., and Birkett, S. (2006). Previous feeding level influences plateau heat production following a 24 h fast in growing pigs. *Br. J. Nutr.* 95, 1082–1087. doi: 10.1079/BJN20061748

Even, P. C., Perrier, E., Aucouturier, J. L., and Nicolaïdis, S. (1991). Utilisation of the method of Kalman filtering for performing the on-line computation of background metabolism in the free-moving, free-feeding rat. *Physiol. Behav.* 49, 177–187. doi: 10.1016/0031-9384(91)90252-J

Johnson, D. E., Johnson, K. A., and Baldwin, R. L. (1990). Changes in liver and gastrointestinal tract energy demands in response to physiological workload in ruminants *J. Nutr.* 120, 649–655.

Kalman, R. E. (1960). A new approach to linear filtering and predictions problems. *J. Basic Eng.* 82, 35–45. doi: 10.1115/1.3662552

Kielanowski, J. (1965). "Estimates of the energy cost of protein depostion in growing animals," in *Energy Metabolism*, ed K. L Blaxter (London: Academic Press), 13–20.

Koong, L. J., Ferrell, C. L., and Nienaber, J. A. (1985). Assessment of interrelationships among levels of intake and production, organ size and fasting heat production in growing animals. *J. Nutr.* 115, 1383–1390.

Koong, L. J., Nienaber, J. A., Pekas, J. C., and Yen, J. T. (1982). Effects of plane of nutrition on organ size and fasting heat production in pigs. *J. Nutr.* 112, 1638–1642.

Kubale, V., Batorek, N., Škrlep, M., Prunier, A., Bonneau, M., Fazarinc, G., et al. (2013). Steroid hormones, boar taint compounds, and reproductive organs in pigs according to the delay between immunocastration and slaughter. *Theriogenology* 79, 69–80. doi: 10.1016/j.theriogenology.2012.09.010

Labussière, E., Maxin, G., Dubois, S., van Milgen, J., Bertrand, G., and Noblet, J. (2009). Effect of feed intake on heat production and protein and fat deposition in milk-fed veal calves. *Animal* 3, 557–567. doi: 10.1017/S1751731108003777

Labussière, E., van Milgen, J., de Lange, C. F. M., and Noblet, J. (2011). Maintenance energy requirements of growing pigs and calves are influenced by feeding level. *J. Nutr.* 141, 1855–1861. doi: 10.3945/jn.111.141291

Le Bellego, L., van Milgen, J., Dubois, S., and Noblet, J. (2001). Energy utilization of low-protein diets in growing pigs. *J. Anim. Sci.* 79, 1259–1271.

Le Goff, G., Dubois, S., van Milgen, J., and Noblet, J. (2002). Influence of dietary fiber level on digestive and metabolic utilization of energy in growing and finishing pigs. *Anim. Res.* 51, 245–259. doi: 10.1051/animres:2002019

Lovatto, P. A., Sauvant, D., Noblet, J., Dubois, S., and van Milgen, J. (2006). Effects of feed restriction and subsequent refeeding on energy utilization in growing pigs. *J. Anim. Sci.* 84, 3329–3336. doi: 10.2527/jas.2006-048

Martin, M. A. (2010). First generation biofuels compete. *N. Biotechnol.* 27, 596–608. doi: 10.1016/j.nbt.2010.06.010

Nelder, J. A., and Mead, R. (1965). A simplex method for function minimization. *Comput. J.* 7, 308–313. doi: 10.1093/comjnl/7.4.308

Noblet, J., Henry, Y., and Dubois, S. (1987). Effect of protein and lysine levels in the diet on body gain composition and energy utilization in growing pigs *J. Anim. Sci.* 65, 717–726.

Noblet, J., Karege, C., Dubois, S., and van Milgen, J. (1999). Metabolic utilization of energy and maintenance requirements in growing pigs: effects of sex and genotype. *J. Anim. Sci.* 77, 1208–1216.

Noblet, J., Shi, X. S., and Dubois, S. (1993). Energy cost of standing activity in sows. *Livest. Prod. Sci.* 34, 127–136. doi: 10.1016/0301-6226(93)90041-F

Ortigues, I., Martin, C., Durand, D., and Vermorel, M. (1995). Circadian changes in energy expenditure in the preruminant calf: whole animal and tissue level. *J. Anim. Sci.* 73, 552–564.

Ortigues, I., Vermorel, M., and Vernet, J. (1994). Calorimétrie indirecte. 1. Calcul des échanges respiratoires des animaux et des humains en vue de la détermination de leurs dépenses énergétiques à l'aide de chambres respiratoires. *Cahier des Techniques de l'INRA* 34, 15–32.

Pekas, J. C., and Wray, J. E. (1991). Principal gastrointestinal variables associated with metabolic heat production in pigs: statistical cluster analyses. *J. Nutr.* 121, 231–239.

Quiniou, N., and Noblet, J. (1995). Prediction of tissular body composition from protein and lipid deposition in growing pigs. *J. Anim. Sci.* 73, 1567–1575.

Quiniou, N., Noblet, J., van Milgen, J., and Dubois, S. (2001). Modelling heat production and energy balance in group-housed growing pigs exposed to low or high ambient temperatures. *Br. J. Nutr.* 85, 97–106. doi: 10.1079/BJN2000217

R Development Core Team. (2010). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Renaudeau, D., Frances, G., Dubois, S., Gilbert, H., and Noblet, J. (2013). Effect of thermal heat stress on energy utilization in two lines of pigs divergently selected for residual feed intake. *J. Anim. Sci.* 91, 1162–1175. doi: 10.2527/jas.2012-5689

Rijnen, M. M. J. A., Verstegen, M. W. A., Heetkamp, M. J. W., and Schrama, J. W. (2003). Effects of two different dietary fermentable carbohydrates on activity and heat production in group-housed growing pigs. *J. Anim. Sci.* 81, 1210–1219.

SAS. (2004). *SAS/STAT® 9.1 User's Guide*. New York, NY: SAS Institute Inc.

Schrama, J. W., Bosch, M. W., Verstegen, M. W., Vorselaars, A. H., Haaksma, J., and Heetkamp, M. J. (1998). The energetic value of non-starch polysaccharides in relation to physical activity in group-housed, growing pigs. *J. Anim. Sci.* 76, 3016–3023.

Soetaert, K., Petzoldt, T., and Setzer, R. W. (2010). Solving differential equations in R: package deSolve. *J. Stat. Softw.* 33, 1–25.

van den Borne, J. J. G. C., Schrama, J. W., Heetkamp, M. J. W., Verstegen, M. W. A., and Gerrits, W. J. J. (2007). Synchronising the availability of amino acids and glucose increases protein retention in pigs. *Animal* 1, 666–674. doi: 10.1017/S1751731107736741

van Milgen, J., Bernier, J. F., Lecozler, Y., Dubois, S., and Noblet, J. (1998). Major determinants of fasting heat production and energetic cost of activity in growing pigs of different body weight and breed/castration combination. *Br. J. Nutr.* 79, 509–517. doi: 10.1079/BJN19980089

van Milgen, J., and Noblet, J. (2000). "Modelling energy expenditure in pigs," in *Modelling Nutrient Utilization in Farm Animals*, eds J. P. McNamara, J. France, and D. E. Beever (Oxon: CAB International), 103–114. doi: 10.1079/9780851994499.0103

van Milgen, J., Noblet, J., Dubois, S., and Bernier, J. F. (1997). Dynamic aspects of oxygen consumption and carbon dioxide production in swine. *Br. J. Nutr.*

78, 397–410. doi: 10.1079/BJN19970159

Vermorel, M., Bouvier, J. C., Bonnet, Y., and Fauconneau, G. (1973). Construction et fonctionnement de deux chambres respiratoires du type "circuit ouvert" pour jeunes bovins. *Ann. Biol. Anim. Bioch. Biophys.* 13, 659–681. doi: 10.1051/rnd:19730409

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 February 2013; accepted: 31 May 2013; published online: 19 June 2013.

Citation: Labussière E, Dubois S, van Milgen J and Noblet J (2013) Partitioning of heat production in growing pigs as a tool to improve the determination of efficiency of energy utilization. Front. Physiol. ***4****:146. doi: 10.3389/fphys.2013.00146*

This article was submitted to Frontiers in Integrative Physiology, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 26 April 2013
doi: 10.3389/fphys.2013.00090

Physical activity and physical activity induced energy expenditure in humans: measurement, determinants, and effects

Klaas R. Westerterp*

Department of Human Biology, Maastricht University Medical Centre, Maastricht, Netherlands

Edited by:
Patrick C. Even, AgroParisTech, France

Reviewed by:
Christy Carter, University of Florida, USA
Schutz Yves, University of Lausanne, Switzerland

****Correspondence:***
Klaas R. Westerterp, Department of Human Biology, Maastricht University Medical Centre, PO Box 616, 6200 MD Maastricht, Netherlands.
e-mail: k.westerterp@maastrichtuniversity.nl

Physical activity is defined as any bodily movement produced by skeletal muscles that results in energy expenditure. The doubly labeled water method for the measurement of total energy expenditure (TEE), in combination with resting energy expenditure, is the reference for physical activity under free-living conditions. To compare the physical activity level (PAL) within and between species, TEE is divided by resting energy expenditure resulting in a figure without dimension. The PAL for sustainable lifestyles ranges between a minimum of 1.1–1.2 and a maximum of 2.0–2.5. The average PAL increases from 1.4 at age 1 year to 1.7–1.8 at reproductive age and declines again to 1.4 at age 90 year. Exercise training increases PAL in young adults when energy balance is maintained by increasing energy intake. Professional endurance athletes can reach PAL values around 4.0. Most of the variation in PAL between subjects can be ascribed to predisposition. A higher weight implicates higher movement costs and less body movement but not necessarily a lower PAL. Changes in physical activity primarily affect body composition and to a lesser extent body weight. Modern man has a similar PAL as a wild mammal of a similar body size.

Keywords: doubly labeled water, accelerometer, age, predisposition, exercise training, energy intake, chronic disease, body composition

INTRODUCTION

Physical activity is defined as any bodily movement produced by skeletal muscles that results in energy expenditure (Caspersen et al., 1985). There are a large number of techniques for the assessment of physical activity ranging from behavioral observation and self-report to motion sensors. The accepted criterion to validate techniques of estimating habitual physical activity, based on the definition of physical activity, is calorimetry. As such, the doubly labeled water method has become the gold standard for the validation of field methods of assessing physical activity. The doubly labeled water method, applied in humans since 1982, is crucial for the measurement of physical activity-induced energy expenditure (AEE) and for the study of determinants and effects.

Physical AEE is determined by body movement and body size. It requires more energy to move a large body than a small body, one of the reasons why obese people generally move less than lean people. Thus, validating field methods of assessing physical activity against energy expenditure requires adjustment for differences in body size. After adjustment for differences in body size, there are clear differences in the level of habitual activity between subjects. Exercise training is the common way to increase the activity level, where professional athletes reach an energy ceiling in endurance exercise.

Determinants and effects of physical activity cannot always be separated. There is a complicated interaction between physical activity and body weight. Body movement requires energy as produced by muscles. Thus, there is an interaction between physical activity, body weight, body composition, and energy expenditure. To move, one uses muscles and energy as stored in body fat. Excess weight in heavier subjects usually implicates excess body fat, limiting weight-bearing activities like running. In addition to body weight and body composition, physical activity is a function of predisposition, age, and environment. There typically are those that are always on the move and those you cannot get on the move. Additionally, physical activity is a function of physical capacity as affected by energy supply and disease.

The current chapter comprises methods for the measurement of physical activity, followed by sections on determinants and effects of physical activity, with a special focus on the doubly labeled water method.

MEASUREMENT OF PHYSICAL ACTIVITY

THE DOUBLY LABELED WATER METHOD FOR THE ASSESSMENT OF TOTAL ENERGY EXPENDITURE

The doubly labeled water method is a method of indirect calorimetry that was introduced for human use about 30 years ago (Schoeller and Van Santen, 1982). The principle of the method is that after a loading dose of water labeled with the stable isotopes of ^{2}H and ^{18}O, ^{2}H is eliminated as water, while ^{18}O is eliminated as both water and carbon dioxide. The difference between the two elimination rates is therefore a measure of carbon dioxide production (**Figure 1**). The deuterium (^{2}H) equilibrates throughout the body's water pool, and the ^{18}O equilibrates in both the water and the bicarbonate pool. The bicarbonate pool consists largely of

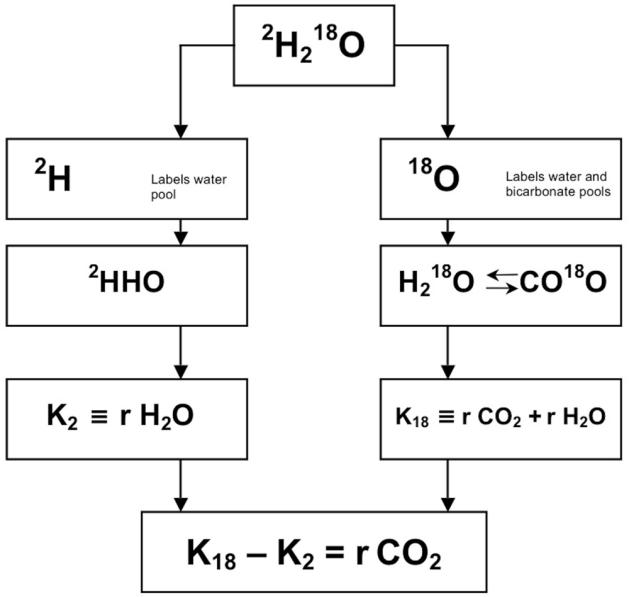


FIGURE 1 | Principle of measurement of carbon dioxide production with doubly labeled water ($^2H_2{}^{18}O$). After administration of water labeled with heavy oxygen (^{18}O) and heavy hydrogen (^2H), the two isotopes mix with the body water, where ^{18}O exchanges with CO_2 in the bicarbonate pools as well. Thus, the elimination rate of ^2H (K_2) is a measure for water loss (rH_2O) and the elimination rate of ^{18}O (K_{18}) is a measure for rH_2O plus carbon dioxide production (rCO_2), and $rCO_2 = K_{18}–K_2$.

dissolved carbon dioxide, which is an end product of metabolism and passes in the blood stream to the lungs for excretion. The rate constants for the disappearance of the two isotopes from the body are measured by mass spectrometric analysis of samples of a body fluid, blood, saliva, or urine.

The method is developed after the discovery in 1949 that the oxygen atoms in the body water and bicarbonate pools are in equilibration. The method was initially used for studying energy metabolism of small animals in the wild. You capture an animal, administer the dose of labeled water, release the animal and then recapture it after an appropriate interval to assess the rate at which the isotopes disappear from the body. One of the first such studies involved measuring the energy cost of a 500-kilometer flight by trained racing pigeons. It was not until 1982 before the method was first used in people. The reason is that ^{18}O-water is expensive and a human requires a much higher dose than a bird. The isotope is not substantially cheaper now, but isotope ratio mass spectrometers have become so sensitive that the method can now work with much smaller doses of isotope. Presently, the method is frequently used with people in several centers.

The method is safe to use in humans as the water is labeled with stable isotopes, ^{18}O and ^2H, at low abundances. Both ^{18}O and ^2H are naturally occurring isotopes, which are present in the body prior to the administration of doubly labeled water. As such, tracer studies depend not on measurement of isotopes concentration, but rather on concentrations in excess of natural abundance or background isotope concentrations. The nominal natural abundances of ^{18}O and ^2H are 2000 and 150 ppm, respectively. Typical doses of doubly labeled water only produce excess isotope abundances of 200–300 and 100–150 ppm for ^{18}O and ^2H, respectively.

The doubly labeled water method can be used to measure carbon dioxide production and hence energy production in free-living subjects for periods of some days to several weeks. The optimal observation period is 1–3 biological half-lives of the isotopes. The biological half-life is a function of the level of the energy expenditure. The optimal observation interval ranges between 3 days for highly active subjects or prematures, respectively, and about 4 weeks in elderly (sedentary) subjects.

An observation starts by collecting a baseline sample. Then, a weighed isotope dose is administered, usually a mixture of 10% ^{18}O and 5% ^2H in, for a 70 kg adult, 100–150 cc water. Subsequently the isotopes equilibrate with the body water and the initial sample is collected. The equilibration time is, depending on body size and metabolic rate, for adults 4-8 h. During equilibration the subject usually does not consume any food or drink. After collecting the initial sample the subject resumes its routines according to the instructions of the experimenter and is asked to collect body water samples (blood, saliva, or urine) at regular intervals until the end of the observation period.

Validation studies resulted in an accuracy of 1–3% and a precision of 2–8%, comparing the method with respirometry. The method has now been applied in subjects at a wide age range and at different activity levels, from premature infants to elderly and from hospitalized patients to participants in a cycle race. The method needs high precision isotope ratio mass spectrometry, working at low levels of isotope enrichment for money reasons mentioned above (Speakman, 1997).

There is still discussion on the ideal sampling protocol, i.e., multi-point versus two-point method. We prefer a combination of both, taking two independent samples at the start, in the midpoint, and at the end of the observation period. Thus an independent comparison can be made within one run, calculating carbon dioxide production from the first samples and the second samples over the first half and the second half of the observation interval (Westerterp et al., 1995).

The doubly labeled water method gives precise and accurate information on carbon dioxide production. Converting carbon dioxide production to energy expenditure needs information on the energy equivalent of CO_2, which can be calculated with additional information on the substrate mixture being oxidized. One option is the calculation of the energy equivalent from the macronutrient composition of the diet. In energy balance, substrate intake and substrate utilization are assumed to be identical. In conclusion, doubly labeled water is an excellent method to measure energy expenditure in unrestrained humans in their normal surroundings over a time period of 1–4 weeks.

TOTAL ENERGY EXPENDITURE, ACTIVITY INDUCED ENERGY EXPENDITURE, AND PHYSICAL ACTIVITY LEVEL

Total energy expenditure (TEE) consists of four components, i.e., the sleeping metabolic rate (SMR), the energy cost of arousal, the thermic effect of food or diet-induced energy expenditure (DEE), and the energy cost of physical activity or AEE. Sometimes daily

FIGURE 2 | Components of total energy expenditure for an average young adult woman and man as adapted from Westerterp et al. (1996).

energy expenditure is divided into three components, taking SMR and the energy cost of arousal together as energy expenditure for maintenance or basal metabolic rate (BMR). BMR is usually the main component of TEE (**Figure 2**).

Activity-induced energy expenditure is derived from TEE minus DEE and BMR: AEE = TEE – DEE – BMR. TEE is measured with doubly labeled water as described in the foregoing section. DEE is assumed to be 10% of TEE in subjects consuming the average mixed diet and being in energy balance (Westerterp, 2004). Thus, AEE can be calculated as: AEE = 0.9 TEE – BMR. BMR is measured or estimated with a prediction equation. A measurement of BMR must meet standard conditions of rest, thermoneutrality, fasting, and immobility. The subject must be awake and the measurement must be performed in a thermoneutral environment to avoid heat production or heat loss for maintenance of body temperature. Furthermore the subject must be in the fasted state (absence of DEE) and in rest (absence of AEE). To meet the conditions in practice, measurement of BMR is performed in the early morning. Subjects are instructed to fast overnight before the BMR measurement, and to transport themselves to the research center in a vehicle or bus. They are also asked to avoid exercise the day before testing. Using a ventilated hood system, BMR is measured for 30 min in the supine position. To eliminate effects of subject habituation to the testing procedure, the respiratory measurements during the first 10 min are discarded, and the following 20 min are used to calculate BMR (Adriaens et al., 2003). Alternatively, BMR is estimated with a prediction equation from height, weight, age and gender like the Schofield equations adopted by the FAO/WHO/UNU (2004).

Activity-induced energy expenditure is the most variable component of TEE. To compare AEE between subjects, AEE should be normalized for differences in body size. A frequently used method is expression of AEE per kg body mass, assuming that expenditure associated with physical activity is weight dependent (Schoeller and Jefford, 2002). For comparison of AEE between children and adolescents, AEE is expressed per kg body mass (Hoos et al., 2003) or per kg fat-free mass (Ekelund et al., 2004). Adjusting AEE for fat-free mass is suggested to remove the confounding effect of sex. To compare the physical activity level (PAL) within and between species TEE in MJ/day is divided by BMR in MJ/day, resulting in a figure without dimension: PAL = TEE/BMR. BMR is determined by body size and composition, age and gender. Dividing TEE by BMR adjusts for specific subject characteristics. A larger subject has higher BMR than a smaller subject. TEE is higher as well, and divided by BMR might result in a comparable PAL to a smaller subject.

LIMITS TO THE PHYSICAL ACTIVITY LEVEL

Data on free-living energy expenditure, as measured with doubly labeled water, permit the evaluation of limits to the PAL. In our site, data were compiled for more than 600 subjects, where energy expenditure was measured over an interval of 2 weeks with the same protocol (Westerterp et al., 1995). The sample excludes individuals aged under 18 years, or those involved in interventions in energy intake, physical activity including athletic performance, or those that were pregnant, lactating or diseased (**Table 1**). The sample includes similar numbers of women and men, with a wide range for age, height, weight, and body mass index. Despite the wide variation in subject characteristics, there is a narrow range of the PAL of the subjects (**Figure 3**).

The PAL for "sustained lifestyles" ranges between 1.1 – 1.2 and 2.0 – 2.5 as suggested earlier by Black et al. (1996). There is no

Table 1 | Characteristics of healthy subjects living in Northern Europe, where the physical activity level is measured over 14 days under free-living conditions with doubly labeled water.

Parameter	Women ($n = 301$)		Men ($n = 346$)	
	Mean ± SD	Range	Mean ± SD	Range
Age (y)	42 ± 19	18 – 96	45 ± 19	18 – 96
Height (m)	1.66 ± 0.07	1.49 – 1.86	1.78 ± 0.07	1.60 – 2.04
Body mass (kg)	72 ± 18	40 – 164	84 ± 18	50 – 216
Body mass index (kg/m^2)	26.2 ± 6.2	14.1 – 55.3	26.4 ± 5.3	15.7 – 61.7
Resting energy expenditure (MJ/d)	6.1 ± 1.0	3.6 – 10.8	7.5 ± 1.1	4.7 – 12.7
Total energy expenditure (MJ/d)	10.3 ± 2.0	4.8 – 18.4	13.2 ± 2.7	6.4 – 22.6
Physical activity level*	1.70 ± 0.23	1.13 – 2.85	1.77 ± 0.28	1.13 – 2.69

**Total energy expenditure as a multiple of resting energy expenditure.*

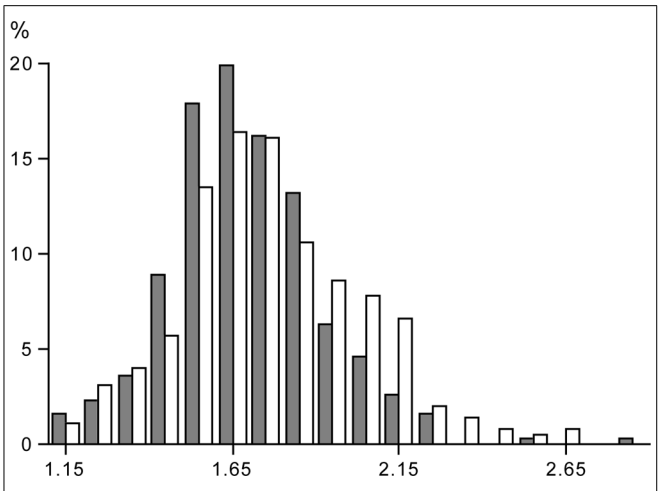


FIGURE 3 | Frequency distribution of the value of the physical activity level, total energy expenditure as a multiple of basal metabolic rate, in a group of women (closed bars) and men (open bars), where subject characteristics are presented in Table 1.

sex difference in the PAL. The minimum value of 1.1 – 1.2 is for a subject with no physical activity, TEE being the sum of BMR and DEE. The maximum value of 2.0 – 2.5 is determined by energy intake (Westerterp, 1998). Higher values are difficult to maintain over a long period of time and generally result in weight loss, unless intake is supplemented (see also the section 3.2).

The PAL of a subject can be classified in three categories as defined by the last FAO/WHO/UNU expert consultation on human energy requirements (2004). The physical activity for sedentary and light activity lifestyles ranges between 1.40 and 1.69, for moderately active or active lifestyles between 1.70 and 1.99, and for vigorously active lifestyles between 2.00 and 2.40.

NON-CALORIMETRIC MEASUREMENT OF PHYSICAL ACTIVITY

There are a large number of non-calorimetric techniques for the assessment of physical activity, which can be grouped into three general categories: behavioral observation, questionnaires (including diaries, recall questionnaires, and interviews), and physiological markers like heart rate and motion sensors. Non-calorimetric techniques of estimating habitual physical activity are needed to study the relationship between physical activity and health. The greatest obstacle to the usage of field methods of assessing physical activity in humans has been the lack of an adequate criterion to which techniques may be compared. The interrelation of various field methods may be of some value, but because there are errors in all methods it is impossible to determine the true validity of any one of them in doing so (Montoye et al., 1996). However, the doubly labeled water method has become the gold standard for the validation of field methods of assessing physical activity (Melanson and Freedson, 1996).

The indicated alternative for doubly labeled water, to assess the PAL of a subject in daily life, is a doubly labeled water validated accelerometer. Accelerometers can be used to study patterns of activity in time. A new generation of accelerometers will provide information on body posture and activity recognition to allow objective assessment of subjects' habitual activities, options for a healthy change, and effects of the follow-up of any changes (Bonomi and Westerterp, 2012). Simultaneous measurement of body acceleration and heart rate can give information on physical fitness (Plasqui and Westerterp, 2006). Behavioral observation and questionnaires, as a self-report method, can be adequately used as an activity-ranking instrument (Westerterp, 2009).

DETERMINANTS AND EFFECTS OF PHYSICAL ACTIVITY

PHYSICAL ACTIVITY LEVEL AND AGE

Young children have a low PAL. Activity energy expenditure increases from 20% at age one to ∼35% at age 18 (Butte et al., 2012). The increase is reflected in the increase of the PAL from 1.4 to 1.75. Activity energy expenditure adjusted for body weight does not show a systematic increase but ranges between about 60 and 80 kJ/kg. It seems young children have a lower activity expenditure and PAL because it takes less energy to move around with a lower body weight. Accelerometers provide information on the activity pattern including activity intensity. Despite the constancy of activity energy expenditure adjusted for body weight from childhood to adulthood, the movement pattern clearly differs. Young children spend more of their active time on high intensity activities (Hoos et al., 2004). Young adults spend on average 9% of their active time on high intensity activities, while the corresponding percentage among the elderly was found to be 4%. In contrast, children spend on average 19% of their total active time on high intensity activities (**Figure 4**). The difference in time spent on high intensity activities between children and adults reflects the different activity patterns among children, which are characterized by short, intermittent bouts of vigorous activity. Probably because of their lower body weight it is easier for children to perform high intensity activities.

Physical activity of an 18-year subject is on average not different from physical activity in a 50-year subject. After age 50, physical activity generally declines, in women as well as men, resulting in a mean PAL of about 1.4 at the age of 90 year (Speakman and

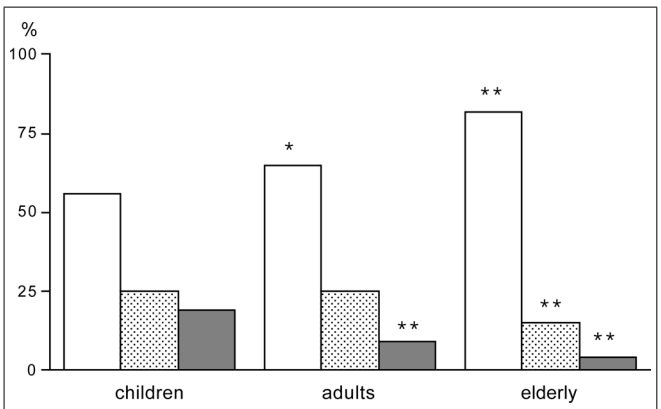


FIGURE 4 | Time spend in activities of low intensity (open bar), moderate intensity (stippled bar) and high intensity (closed bar), expressed as a percentage of the active time, for children, adults and elderly; *$P < 0.05$ for difference between adults and children; and **$P < 0.01$ for difference between adults or elderly and children (After Hoos et al., 2004).

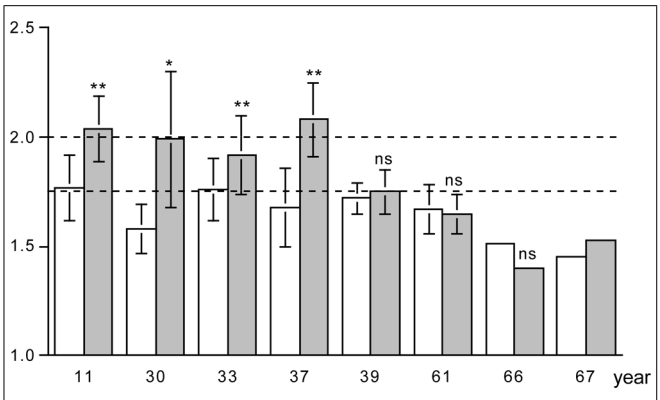


FIGURE 5 | The physical activity level, total energy expenditure as a multiple of basal energy expenditure, before (open bar) and at the end of a training program (closed bar), for eight studies displayed in a sequence of age of the participants as indicated on the horizontal axis. The horizontal broken lines denote the average physical activity level of 1.75 and the ceiling value of 2.00 for non-athletes; * $P < 0.05$; and ** $P < 0.01$ for difference with before training program (After Bingham et al., 1989; Blaak et al., 1992; Goran and Poehlman, 1992; Westerterp et al., 1992; Kempen et al., 1995; Van Etten et al., 1997; Hunter et al., 2000; Meijer et al., 2001).

Westerterp, 2010). A PAL of 1.4 is the same as the average PAL for a subject staying in a respiration chamber (Westerterp and Kester, 2003). It seems logical that the PAL of a 90-year old is comparable to the PAL for a subject staying all day in a chamber. At age 90, one does not go out very often anymore. The activity pattern of elderly subjects is characterized by low intensity activities (Meijer et al., 2001).

In conclusion, it seems physical activity is the highest at reproductive age.

PHYSICAL ACTIVITY LEVEL AND EXERCISE TRAINING

There is a limited number of exercise training studies where the PAL was measured with doubly labeled water, before and at the end of the training intervention. Combining the data of the studies by plotting the PAL in a sequence of the age of the subjects, there are some clear observations to make (**Figure 5**). The PAL before training ranges from lower values around 1.5 in elderly subjects to moderate values around 1.75 in younger subjects. Exercise training induces an increase in physical activity in younger subjects but not in older subjects. The exception is a training study in 39-year subjects; however, in this study training was combined with energy restriction to induce weight loss. In younger subjects, the mean physical activity values reached a ceiling value around 2.0. No training study reported individual PAL values over 2.5. Thus, exercise training induces an increase in physical activity when one is young or middle-aged and eats *ad libitum*.

The lack of an effect of exercise training on the physical activity can only be explained by a compensatory reduction of physical activity in the non-training time. Observations with accelerometers have shown imposed exercise training did not influence spontaneous activity in younger subjects so that their total PALs increased (Meijer et al., 1991; Van Etten et al., 1997). In contrast, elderly subjects compensate for exercise training by a decline in spontaneous physical activity, so that PALs remain unchanged (Meijer et al., 1999).

A potential explanation for a compensatory reduction of physical activity in the non-training time is a negative energy balance. PAL did not increase when exercise training was combined with an energy-restricted diet (Kempen et al., 1995). The PAL in elderly subjects might not respond to exercise training because of a limitation through energy intake, as indicated by a study of the effect of age on energy balance (Ainsli et al., 2002). Exposing 24-year and 56-year subjects to the same strenuous hill walking activity for 10 days resulted in a similar expenditure of about 21.5 MJ/d, where energy intake in the young subjects was with 19.2 MJ/d close to expenditure while the older subjects ate 4 MJ/d less.

The PAL reaches a maximum value of 2.5 times resting energy expenditure in non-athletes. However, professional endurance athletes can reach a value around 4.0 and can maintain this values for several weeks (Westerterp et al., 1986). They are a selection of the population, born to be athletes, training for many years to reach their high level of performance. The training includes exercise and the maintenance of energy balance at a high level of energy turnover. The latter implicates the supplementation of the diet with energy drinks. Highly trained athletes have learned to eat the maximum amount of food during hard physical work (Sjödin et al., 1994).

PHYSICAL ACTIVITY AND PREDISPOSITION

Some can quietly sit and read for hours while others do not have the perseverance to be quiet. The "between subjects variation" in physical activity is large as reflected in the doubly labeled water assessed PAL under daily life conditions in non-athletes (**Figure 3**). Surprisingly, between subjects variation in physical activity is also large within the identical confined space of a respiration chamber, indicating an effect of predisposition (Westerterp and Kester, 2003). The mean PAL of the subjects in the chamber was 1.40 ± 0.06, on the lower end of the frequency distribution (**Figure 3**) as expected. However, the minimum value was as low as 1.30 and the maximum value as high as 1.58. There was a subject with an AEE of 1.0 MJ/d and a similar sized subject spending 3.0 MJ/d in AEE. Subjects with a relatively low or high PAL in the respiration chamber turned out to be, respectively, relatively sedentary or physically active in free-living conditions as well (**Figure 6**). Further studies, as described below, provided evidence for an important genetic component in the threefold variation in AEE among individuals in the same confined environment of a respiration chamber and the significant relation with PAL in free-living conditions.

The test for a genetic contribution was based on a classic twin design. Intrapair differences in monozygotic twins are due to environmental factors and measurement errors, whereas intrapair differences in dizygotic twins are additionally affected by genetic factors. Physical activity was measured over two consecutive weeks with a doubly labeled water validated tri-axial accelerometer for the measurement of movement. Subjects were 20 same-sex twin pairs, including similar numbers of monozygotic and dizygotic twins, age 25 ± 7 year, and not living together. The PAL was significantly related within twin pairs and the relation was nearly twice as strong within monozygotic than within dizygotic twins. The calculated contribution of genetic factors to the variance in

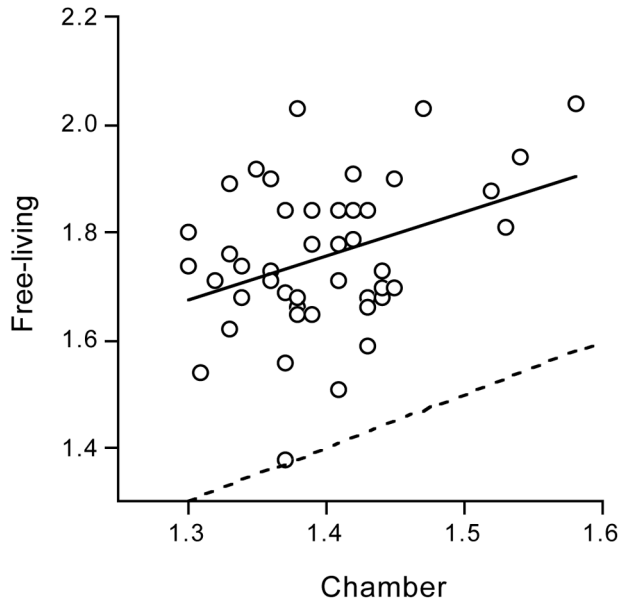


FIGURE 6 | Free-living physical activity level plotted as a function of physical activity level in the confined environment of a respiration chamber, with the line of identity (dotted) and the linear regression line (continuous) (After Westerterp and Kester, 2003).

physical activity was 72–78% (Joosen et al., 2005). Thus, a large part of the variation in physical activity between subjects can be ascribed to predisposition. The relatively high contribution of a genetic component to variation in physical activity does not automatically imply subjects with high predisposition for a sedentary life style are less active than subjects with a predisposition for an active life style. The ultimate activity level is the outcome of an interaction between genes and environment. It only takes more effort for subjects with a predisposition for a sedentary life style to reach the same activity level as for those with predisposition for an active life style.

PHYSICAL ACTIVITY LEVEL AND BODY WEIGHT

Physical activity implies displacement of the weight of a body part like arms, legs, or the full body. Together with activity duration and intensity, body weight determines the variation in AEE. The effect of body weight on physical activity is illustrated by activity changes during growth from birth to adult weight, physical activity and underweight in anorectic subjects, and physical activity and overweight in obese subjects. Body weight increases from three to four kg at birth to an adult value of 60 to 70 kg. Activity energy expenditure adjusted for body weight does not show a systematic increase, as explained in section 3.1. Children can spend more of the active time in high intensity activities than adults (**Figure 4**), as it takes less energy to move the smaller body.

In adults, underweight and overweight is often associated with hyperactivity and hypo-activity, respectively. By monitoring body movement in addition to the measurement of TEE with doubly labeled water, it was shown the paradoxical hyperactivity in anorexia nervosa only occurs in subjects with a higher body mass index (Bouten et al., 1996). The average PAL was not different between a group of women with anorexia nervosa and a control group. However, when subjects were assigned to low, moderate and high levels of daily physical activity, a u-shaped distribution was found for the women with anorexia while control subjects were normally distributed with respect to different activity levels. The u-shaped distribution in women with anorexia was related to the body mass index of the subjects, with relatively low body mass index values corresponding to low levels of physical activity and high body mass index values corresponding to high levels of physical activity. Subjects with a relatively low body mass index had low levels of physical activity and spent less time on activities like sports and exercise, and more time on activities like standing, lying, or sitting than subjects with a higher body mass index. This is in accordance with the reduction in physical activity in the course of chronic energy deficiency and human starvation (see section 3.5). Physical activity decreases as a consequence of malnutrition and declining physical capacity.

Overweight and obesity is not associated with a lower PAL. Activity energy expenditure is similar or even higher in heavier subjects. Only in subjects with a body mass index higher than 35 kg/m^2, PAL values are reduced (Prentice et al., 1996). Selecting young adults, age range 18–50 year, from our own database as presented in **Table 1**, leads to the same conclusion (**Figure 7**). The average PAL is around 1.75 for all body mass index categories except the very highest. The average value for subjects with a body mass index of 40 kg.m^2 or higher ($n = 12$) was 1.65 ± 0.24.

A study in adolescents from the same school showed AEE was similar for obese and gender matched control subjects (Ekelund et al., 2002). The fact that AEE is similar and not proportionally higher in subjects with a higher body weight has consequences for body movement. Indeed body movement, as measured simultaneously with accelerometers, was lower in obese than in normal-weight subjects. Overweight implies less physical activity, that is less body movement, but because of the larger body weight, the decreased movement still results in similar energy expenditure as subjects with a normal bodyweight. In conclusion, a higher weight implies less body movement as shown by the typical occurrence of high intensity activity bursts in young children before reaching adult weight. Overweight subjects are

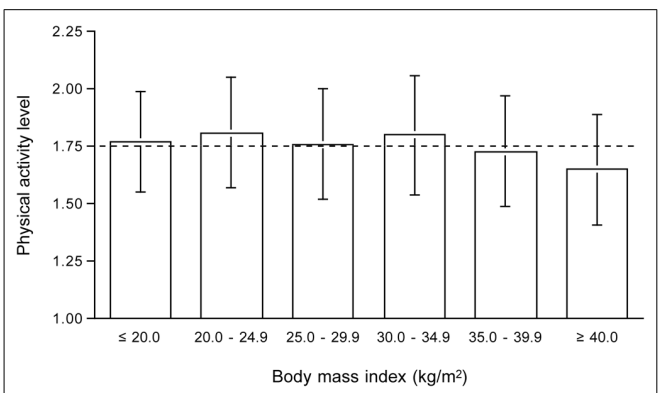


FIGURE 7 | Physical activity level by body mass index category for subjects aged 18 to 50 year from Table 1. The horizontal broken line denotes the average physical activity level of 1.75.

less physically active than normal-weight subjects despite physical activity-related energy expenditure is not necessarily lower.

PHYSICAL ACTIVITY AND ENERGY INTAKE

There are several studies on the effect of overfeeding and underfeeding on physical activity as measured under free-living conditions with doubly labeled water. The effect of overfeeding on physical activity, calculated by expressing TEE as a multiple of resting energy expenditure is non-significant (Westerterp, 2010). There does not seem to be an effect of overfeeding on physical activity, when overfeeding is lower than twice maintenance requirement, as observed in studies lasting up to 9 weeks.

Long-term underfeeding clearly affects physical activity as already shown by the Minnesota experiment (Keys et al., 1950). It was initiated to determine the effects of relief feeding, necessitated by the famine in occupied areas of Europe during World War II. Normal weight men were subjected to 24-weeks of semi-starvation, followed by rehabilitation. The weight maintenance diet of 14.6 MJ/d was reduced to 6.6 MJ/d during semi-starvation. In the 24 weeks of semi-starvation, body weight went down from an average of 69 to 53 kg. At the end of the 24-week interval, subjects reached a new energy balance as body weight leveled off at the lower value. Energy expenditure equalled energy intake, i.e., energy expenditure went down from 14.6 MJ/d to 6.6 MJ/d, a reduction of 55%. The largest saving on energy expenditure could be ascribed to a decrease in activity energy expenditure (**Table 2**). Subjects were not capable of doing anything more than hanging around. More recent underfeeding studies were generally performed in overweight and obese subjects, not reducing body weight as much below normal values as in the Minnesota experiment. Then, underfeeding does not seem to affect PAL though there are indications for a reduction, not persisting in time (Westerterp, 2012).

There are many comparative studies on the effect underfeeding and the effect of underfeeding in combination with exercise training. The general conclusion is that underfeeding is an effective method to lose weight and that there is little effect of an additional exercise-training program. Explanations for a non-existent effect of the addition of exercise to an energy-restricted diet are a low compliance to the exercise prescription and/or a negative effect of exercise training on dietary compliance. Another explanation for a non-existent effect on weight loss of the addition of exercise to an energy-restricted diet is derived from a typical study performed in Maastricht (Kempen et al., 1995). Obese women were randomly assigned to diet alone or diet and exercise for 8 weeks. The exercise group participated in aerobic and fitness exercises, in three 90-min sessions per week, supervised by a professional trainer. Daily energy expenditure decreased similarly in the diet group and the diet plus exercise group from 12.3 to 10.8 MJ/d and from 12.1 to 11.0 MJ/d, respectively. The PAL was the same for the two groups, before as well as at the end of the intervention. Exercise training did not induce an increase in AEE as observed in subjects with *ad libitum* food intake. Subjects compensated for the training activity with a decrease in physical activity during the non-training time.

PHYSICAL ACTIVITY LEVEL AND DISEASE

Chronic disease negatively affects physical activity, here illustrated by observations in patients with chronic obstructive pulmonary disease (COPD). COPD is associated with muscle wasting, a decrease in respiratory muscle strength and endurance and impaired physical fitness. Patients with COPD often suffer from weight loss due to an inadequate dietary intake combined with increased energy expenditure. Physical activity, as the main determinant of variation in energy requirement, may play an important role. TEE in COPD is elevated, which can be primarily attributed to the activity component. Interestingly, there is no difference in TEE between COPD patients with normal resting energy expenditure and those with increased resting energy expenditure (Baarends et al., 1997). Patients with normal resting energy expenditure appeared to have higher energy expenditure for activities than those patients with COPD who had increased resting energy expenditure. The PAL was significantly higher in the former group than in the latter group. Physical activity affects the energy need of the COPD patient and determines energy balance. In depleted ambulatory outpatients with COPD, energy balance could be reached with oral nutritional supplements as a function of physical activity. Weight change was negatively associated with the energy requirement for physical activity (**Figure 8**). Patients with a PAL above 1.55 lost weight and with a PAL below 1.55 gained weight (Goris et al., 2003). The disease appears to be an important limitation for an active lifestyle. Chronic disease reduces physical activity and physical capacity, possibly through a limited energy supply.

PHYSICAL ACTIVITY AND BODY COMPOSITION

The interrelation between physical activity and body composition is based on comparisons between subjects and within subjects. In a between subject design, body composition is compared between subjects with a lower and higher activity level. The question is whether body composition differs between sedentary and physically active individuals. In a within subject design, body composition is compared within subjects before and after an activity intervention. Then, the question is whether body composition changes when one gets less active or more active. Both analyses are

Table 2 | Energy saved by 24 weeks semi-starvation in the Minnesota Experiment (Keys et al., 1950).

	MJ/d	% of total	
Basal metabolic rate	2.6	32	65% for decreased active tissue; 35% for lower tissue metabolism
Diet-induced energy expenditure	0.8	10	
Activity-induced energy expenditure	4.7	58	40% for reduced body weight; 60% for reduced physical activity
Total	8.0	100	

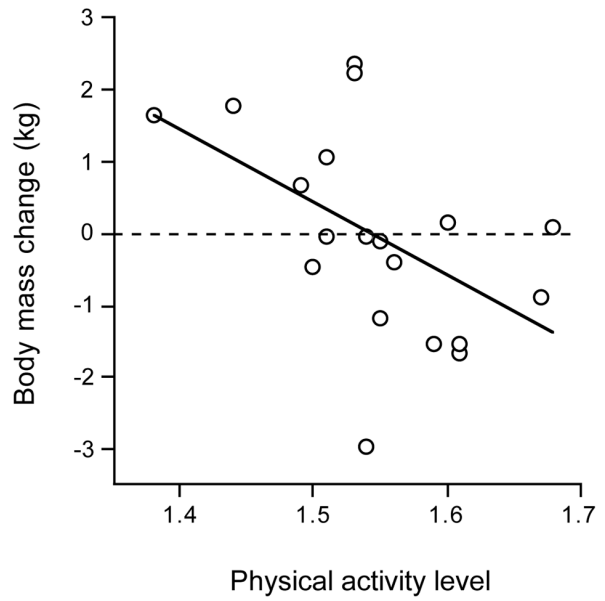


FIGURE 8 | Body mass change in patients with chronic obstructive pulmonary disease over three months after clinical rehabilitation, plotted as a function of the physical activity level (After Goris et al., 2003).

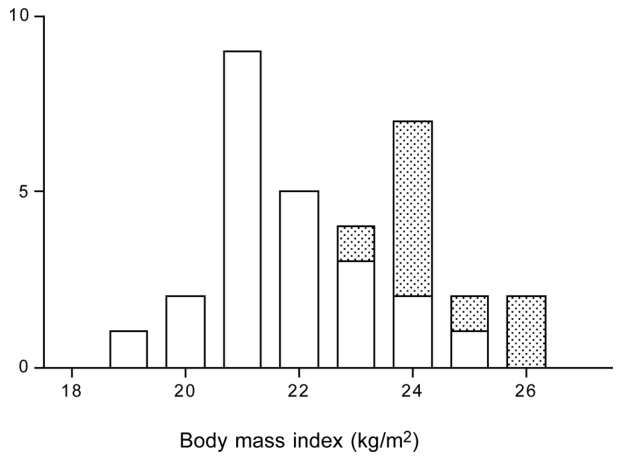


FIGURE 9 | Frequency distribution of the body mass index of subjects that successfully trained to run a half marathon (open bars) and of the dropouts (stippled bars), the latter were 9 out of 32 subjects (After Westerterp et al., 1992).

described; starting with a comparison between subjects followed by a description of the effect of changes in activity behavior on body composition within the same individual.

The comparison of body composition between subjects with a lower and higher activity level was conducted in a cohort of 529 subjects, included in the compiled data presented in **Table 1** (Speakman and Westerterp, 2010). The analysis showed that at the population level, differences in body composition are generally not related to differences in physical activity. Increasing age is associated with a lower PAL, higher fat mass and lower fat-free mass. For the same body weight, body composition is different at older ages than at younger ages, i.e., fat mass is higher and fat-free mass is lower in the elderly. However, the age-induced reduction of physical activity does not seem to be directly related to the age-induced increase in fat mass and decrease in fat-free mass. At any age, body mass does not systematically differ between a sedentary and a more physically active subject.

Many studies show changes in body composition in response to a change in physical activity through exercise training. In young adults, long-term endurance training induces an increase in fat-free mass and, when available, a decrease in fat mass. The latter effect is especially pronounced in men. Here, as an example, a training study in 37-year subjects as included in **Figure 5**. The study included a training program of nearly 1 year in preparation of running a half marathon (Westerterp et al., 1992). Subjects were sedentary men and women who did not participate in any sport like running or jogging and who were not active in any other sport for more than 1h/week. Out of nearly 400 respondents to an advertisement, 16 women and 16 men were selected, between the ages of 30–40 years old, with a normal body weight. The latter implied a body mass index, based on self reported weight and height, between 20 and 25 kg/m^2. During the study, five women and four men withdrew because they were unable to keep up with the training program. It appeared all dropouts were in the heaviest category with a body mass index of 23 kg/m^2 or higher (**Figure 9**). The observation implies that it is difficult to keep up high-intensity training with a higher body weight, especially training involving weight displacement like running. Surprisingly, successful subjects did not lose weight. Apparently, the exercise training-induced increase in energy requirement eventually increased hunger. One has to eat more to maintain the additional training activity, especially in the long-term. The 11 women finishing the 40-week training lost on average 2 kg fat and gained 2 kg fat-free mass. The 12 men that finished the training lost on average 4 kg fat and gained 3 kg fat-free mass. For men, the change in body fat was highly related to the initial fat mass. That is, subjects with a higher initial percentage body fat lost more fat than those who were leaner at the start. This was not so for women (**Figure 10**). Body fat can be reduced by physical activity although women tend to compensate more for the increased energy expenditure with an increased intake, resulting in a smaller effect compared with men. Women tend to preserve their energy balance more closely than men. Women especially do not lose much body fat, even when a high exercise level can be maintained.

The increase in fat mass with increasing age is not prevented through a physically active lifestyle (Westerterp and Plasqui, 2009). Young adults were observed over an average time interval of more than 10 years. Physical activity was measured over two-week periods with doubly labeled water and doubly labeled water validated tri-axial accelerometers, and body fat gain was measured with isotope dilution. There was a significant association between the change in physical activity and the change in body fat, where subjects with higher activity level at the start were those with a higher fat gain at follow up after more than 10 years. A physically active lifestyle inevitably results in a larger decrease of daily energy expenditure at later age than a sedentary lifestyle. A change to a

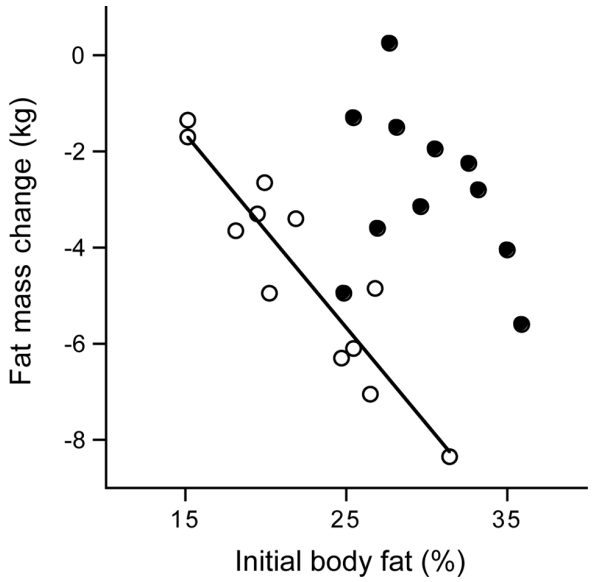


FIGURE 10 | Fat mass change from before until 40 weeks after the start of a training period to run a half marathon plotted versus the initial body fat percentage for women (closed dots) and men (open dots) with the calculated linear regression line for men (After Westerterp et al., 1992).

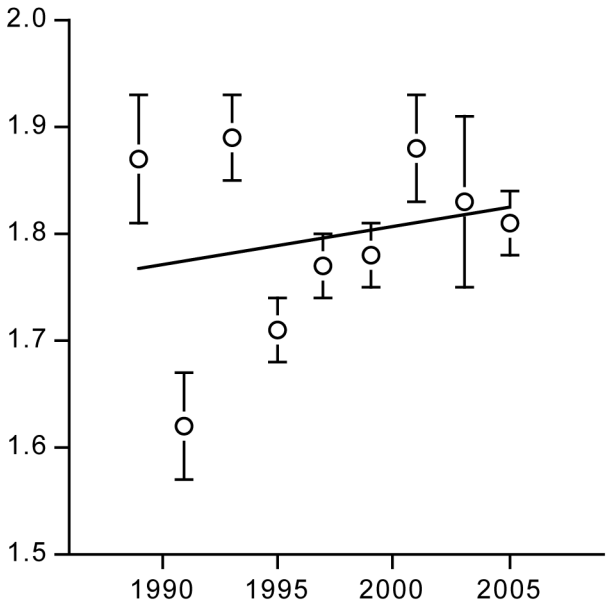


FIGURE 11 | Time trend of the physical activity level for a population around Maastricht in the Netherlands (After Westerterp and Speakman, 2008).

more sedentary routine does not induce an equivalent reduction of energy intake, even in the long-term, and most of the excess energy is stored as fat. Thus, it seems difficult to overcome the loss of fat-free mass and the gain of fat mass with increasing age.

PHYSICAL ACTIVITY LEVEL OF MODERN MAN

Energy expenditure of modern man is generally thought to be low. People increasingly adopt sedentary lifestyles in which motorized transport, mechanized equipment, and domestic appliances displace physical activities and manual work. Few people are employed in active occupations and leisure time is dominated by sedentary activities behind a computer or watching television. On the other hand, the main part of variation in physical AEE between individuals can be ascribed to genetics, as described in section 3.3. It is unlikely that the genetic background has changed. Changes through natural selection take tens of generations, especially for features like physical activity, determined by many genes. Additionally, in the current society with an abundant food supply, there is no selection pressure in favor of a low physical activity, i.e., low energy expenditure, that otherwise would be necessary to limit energy requirement.

The PAL of modern man is put in perspective, based on analysis of measurements with doubly labeled water (Westerterp and Speakman, 2008). Three tests were performed. Firstly, changes in PAL, as derived from TEE and resting energy expenditure, were compiled over time (**Table 1**). Secondly, PAL in modern Western societies were compared with those from third world countries mirroring the physical activity in Western societies in the past. Thirdly, levels of physical activity of modern humans were compared with those of wild terrestrial mammals, taking into account body size and temperature effects.

The PAL slightly increased over time (**Figure 11**), indicating physical activity did not decrease during the two decades where rates of obesity doubled in the Netherlands. Compiled literature data from North America, where obesity rates tripled over the same time interval, also suggested the PAL increased rather than decreased. PAL in rural third world countries were not different from individuals of Western societies.

The doubly labeled water method started with studying energy metabolism of animals in the wild. Since then, data on more than 90 different terrestrial mammal species have been published. Body sizes range from 20-gram mice to wild red deer weighing over 100 kg. For many wild mammals measures of TEE are made at ambient temperatures below the thermoneutral zone. Thus, the PALs for these mammals reflect the combination of activity expenditure and the energy spent on thermoregulation. In fact the PAL calculated as TEE divided by basal energy expenditure is negatively related to body weight (**Figure 12**) reflecting the increasing thermoregulatory load as body size declines. Hence the PAL for contemporary humans is at the lower end of the distribution of activity level values when the effects of body mass are ignored, in line with the previous findings, but they are at exactly the expected level, once the effect of body weight on the PAL is taken into account.

In conclusion, a free-living mammal close to the body size of man has a comparable activity level to humans. The PAL of modern man is in line with a free-living wild mammal.

DISCUSSION

Physical activity, defined as any bodily movement produced by skeletal muscles that results in energy expenditure, is derived from measurement of energy expenditure. Doubly labeled water is an

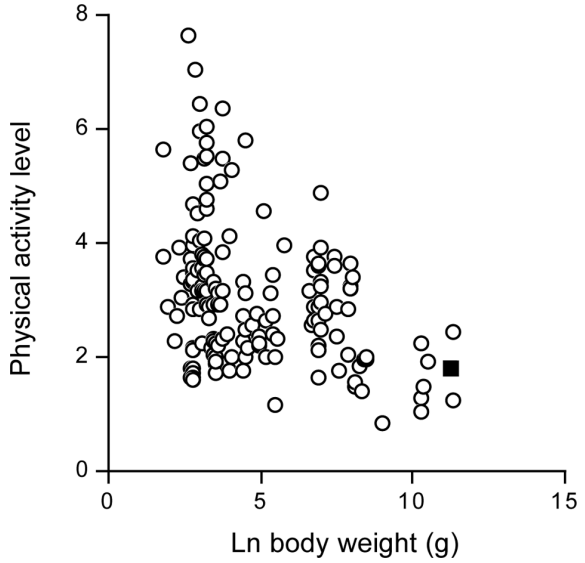


FIGURE 12 | The physical activity level in wild terrestrial mammals, plotted as a function of body weight. The value for modern man is indicated as a closed square (After Westerterp and Speakman, 2008).

excellent method to measure energy expenditure in unrestrained humans over a time period of 1-4 weeks. AEE and PAL is derived from TEE and measured or estimated BMR as described in section 2.2. Alternatively, physical activity can be derived from the residual of the regression of TEE on total body water, where total body water is derived from the dilution spaces of deuterium and O^{18}. BMR is a function of fat-free mass and total body water is a measure for fat-free mass. Thus, differences in total body water reflect differences in BMR.

Activity-induced energy expenditure is the most variable component of TEE and is determined by body size and body movement. The effect of body size on AEE is corrected for by expressing AEE per kg body mass or by expressing TEE as a multiple of BMR. The expression of TEE as a multiple of BMR is precluded when the relation between TEE and BMR has a non-zero intercept (Carpenter et al., 1995). Then, TEE can be adjusted for the effect of body size in a linear regression analysis.

The indicated non-calorimetric method to assess physical activity is a doubly labeled water validated accelerometer (section 2.4). Validation studies of accelerometers with doubly labeled TEE as a reference should be critically evaluated. The largest component of TEE is BMR, as shown by the frequency distribution of PAL values in **Figure 3**, where most PAL values are below 2.5. A PAL value below 2.5 denotes AEE is less than 50% of TEE (Westerterp, 2003). BMR, as the largest component of TEE, can be estimated from height, weight, age, and gender. Thus, prediction equations of TEE based on height, weight, age, and gender usually show a high explained variation. Adding accelerometer output to the equation as an independent variable, often does not explain any additional variation (Plasqui and Westerterp, 2007). The indicator for the validity of an accelerometer is the increase in explained variation or the partial correlation for accelerometer output, not always presented.

Evidence was presented for age, exercise training, predisposition, body weight, energy intake, and disease as determinants of PAL. A decrease of physical activity with increasing age and an increase of physical activity with exercise training affect body composition and to a lesser extent body weight. The fact that a free-living mammal, close to the body size of man, has a comparable level of energy turnover, i.e., a comparable level of physical AEE to humans, indicates that the energy we spend on physical activity lies in the normal range. It may well be that obese individuals seem to behave rather sedentary, but as soon as their weight-bearing activity takes place, they spend a very large amount of energy on activity because of their well known large bearing of body weight.

REFERENCES

Adriaens, M. P. E., Schoffelen, P. F. M., and Westerterp, K. R. (2003). Intra-individual variation of basal metabolic rate and the influence of physical activity before testing. *Br. J. Nutr.* 90, 419–423.

Ainsli, P. N., Campbell, I. T., Frayn, K. N., Humphreys, S. M., MacLaren, D. P. M., Reilly, T., et al. (2002). Energy balance, metabolism, hydration, and performance during strenuous hill walking: the effect of age. *J. Appl. Physiol.* 93, 714–723.

Baarends, E. M., Schols, A. M. W. J., Westerterp, K. R., and Wouters, E. F. M. (1997). Total daily energy expenditure relative to resting energy expenditure in clinically stable patients with COPD. *Thorax* 52, 780–785.

Bingham, S. A., Goldberg, G. R., Coward, W. A., Prentice, A. M., and Cummings, J. H. (1989). The effect of exercise and improved physical fitness on basal metabolic rate. *Br. J. Nutr.* 61, 155–173.

Blaak, E. E., Westerterp, K. R., Bar-Or, O., Wouters, L. J. M., and Saris, W. H. M. (1992). Effect of training on total energy expenditure and spontaneous activity in obese boys. *Am. J. Clin. Nutr.* 55, 777–782.

Black, A. E., Coward, W. A., Cole, T. J., and Prentice, A. M. (1996). Human energy expenditure in affluent societies: an Analysis of 574 doubly-labelled water measurements. *Eur. J. Clin. Nutr.* 50, 72–92.

Bonomi, A. G., and Westerterp, K. R. (2012). Advances in physical activity monitoring and lifestyle interventions in obesity: a review. *Int. J. Obes.* 36, 167–177.

Bouten, C. V. C., Van Marken Lichtenbelt, W. D., and Westerterp, K. R. (1996). Influence of body mass index on daily physical activity in anorexia nervosa. *Med. Sci. Sports Exerc.* 28, 967–973.

Butte, N. F., Ekelund, U., and Westerterp, K. R. (2012). Assessing physical activity using wearable monitors: measures of physical activity. *Med. Sci. Sports Exerc.* 44(Suppl. 1), S5–S12.

Carpenter, W. H., Poehlman, E. T., O'Connell, M., and Goran, M. L. (1995). Influence of body composition on resting metabolic rate on variation in total energy expenditure: a meta-analysis. *Am. J. Clin. Nutr.* 61, 4–10.

Caspersen, C. J., Powell, K. E., and Christenson, G. M. (1985). Physical activity, exercise and physical fitness: definitions and distinctions for health-related research. *Public Health Rep.* 100, 126–131.

Ekelund, U., Aman, J., Yngve, A., Renman, C., Westerterp, K., and Sjöström, M. (2002). Physical activity but not energy expenditure is reduced in obese adolescents: a case–control study. *Am. J. Clin. Nutr.* 76, 935–941.

Ekelund, U., Yngve, A., Brage, S., Westerterp, K., and Sjøstrøm, M. (2004). Body movement and physical activity energy expenditure in children and adolescents: how to adjust for differences in body size and age. *Am. J. Clin. Nutr.* 79, 851–856.

FAO/WHO/UNU. (2004). Human energy requirements. *Rome: FAO Food and nutrition report series* 1.

Goran, M. I., and Poehlman, E. T. (1992). Endurance training does not enhance total energy expenditure in healthy elderly persons. *Am. J. Physiol.* 263, E950–E957.

Goris, A. H. C., Vermeeren, M. A. P., Wouters, E. F. M., Schols, A. M. W. J., and Westerterp, K. R. (2003). Energy balance in depleted ambulatory patients with chronic obstructive pulmonary disease; the effect

of physical activity and oral nutritional supplementation. *Br. J. Nutr.* 89, 725–729.

Hoos, M. B., Gerver, W. J. M., Kester, A. D., and Westerterp, K. R. (2003). Physical activity levels in children and adolescents. *Int. J. Obes. Relat. Metab. Disord.* 27, 605–609.

Hoos, M. B., Kuipers, H., Gerver, W. J. M., and Westerterp, K. R. (2004). Physical activity pattern of children assessed by tri-axial accelerometry. *Eur. J. Clin. Nutr.* 58, 1425–1428.

Hunter, G. R., Wetzstein, C. J., Fields, D. A., Brown, A., and Bamman, M. M. (2000). Resistance training increases total energy expenditure and free-living physical activity in older adults. *J. Appl. Physiol.* 89, 977–984.

Joosen, A. M. C. P., Gielen, M., Vlietinck, R., and Westerterp, K. R. (2005). Genetic analysis of physical activity in twins. *Am. J. Clin. Nutr.* 82, 1253–1259.

Kempen, K. P. G., Saris, W. H. M., and Westerterp, K. R. (1995). Energy balance during 8 weeks energy-restrictive diet with and without exercise in obese females. *Am. J. Clin. Nutr.* 62, 722–729.

Keys, A., Brozek, J., Henschel, A., Mickelsen, O., and Taylor, H. L. (1950). The *Biology of Human Starvation.* Minneapolis: University of Minnesota Press.

Meijer, E. P., Goris, A. H. C., Wouters, L., and Westerterp, K. R. (2001). Physical activity as a determinant of the physical activity level in the elderly. *Int. J. Obes. Relat. Metab. Disord.* 25, 935–939.

Meijer, E. P., Westerterp, K. R., and Verstappen, F. T. J. (1999). The effect of exercise training on total daily physical activity in the elderly. *Eur. J. Appl. Physiol.* 80, 16–21.

Meijer, G. A. L., Janssen, G. M. E., Westerterp, K. R., Verhoeven, F., Saris, W. H. M., and Ten Hoor, f. (1991). The effect of a 5-month endurance-training programme on physical activity; evidence for a sex-difference in the metabolic response to exercise. *Eur. J. Appl. Physiol.* 62, 11–17.

Melanson, E. L., and Freedson, P. S. (1996). Physical activity assessment: a review of methods. *Crit. Rev. Food Sci. Nutr.* 36, 385–396.

Montoye, H. J., Kemper, H. C. G., Saris, W. H. M., and Washburn, R. A. (1996). *Measuring Physical Activity and Energy Expenditure.* Champaign: Human Kinetics.

Plasqui, G., and Westerterp, K. R. (2006). Accelerometry and heart rate monitoring as a measure of physical fitness: cross-validation. *Med. Sci. Sports Exerc.* 38, 1510–1514.

Plasqui, G., and Westerterp, K. R. (2007). Physical activity assessment with accelerometers: an evaluation against doubly labeled water. *Obestity* 15, 2371–2379.

Prentice, A. M., Black, A. E., Coward, W. A., and Cole, T. (1996). Energy expenditure in overweight and obese adsults in affluent societies: an analysis of 319 doubly-labelled water measurements. *Eur. J. Clin. Nutr.* 50, 93–97.

Schoeller, D. A., and Jefford, G. (2002). Determinants of the energy costs of light activities: inferences for interpreting doubly labelled water data. *Int. J. Obes. Relat. Metab. Disord.*26, 97–101.

Schoeller, D. A., and Van Santen, E. (1982). Measurement of energy expenditure in humans by doubly labeled water method. *J. Appl. Physiol.* 53, 955–959.

Sjödin, A., Andersson, A., Högberg, J., and Westerterp, K. (1994). Energy balance in cross country skiers. A study using doubly labeled water and dietary record. *Med. Sci. Sports Exerc.* 26, 720-724.

Speakman, J. R. (1997). *Doubly Labelled Water, Theory and Practice.* London: Chapman and Hall.

Speakman, J. R., and Westerterp, K. R. (2010). Associations between energy demands, physical activity and body composition in adult humans between 18 and 96 years of age. *Am. J. Clin. Nutr.* 92, 826–834.

Van Etten, L. M. L. A., Westerterp, K. R., Verstappen, F. T. J., Boon, B. J. B., and Saris, W. H. M. (1997). Effect of an 18-wk weight-training program on energy expenditure and physical activity. *J. Appl. Physiol.* 82, 298–304.

Westerterp, K. R. (1998). Alterations in energy balance with exercise. *Am. J. Clin. Nutr.* 68, 970S–974S.

Westerterp, K. R. (2003). Impacts of vigorous and non-vigorous activity on daily energy expenditure. *Proc. Nutr. Soc.* 62, 645–650.

Westerterp, K. R. (2004). Diet induced thermogenesis. *Nutr. Metab.* 1, 1–5.

Westerterp, K. R. (2009). Assessment of physical activity: a critical appraisal. *Eur. J. Appl. Physiol.* 105, 823–828.

Westerterp, K. R. (2010). Physical activity, food intake and body weight regulation: insights from doubly labeled water studies. *Nutr. Rev.* 68, 148–154.

Westerterp, K. R. (2012). Metabolic adaptations to over- and underfeeding – still a matter of debate? *Eur. J. Clin. Nutr*. doi: 10.1038/ejcn.2012.2012.187 [Epub ahead of print].

Westerterp, K. R., and Kester, A. D. M. (2003). Physical activity in confined conditions as an indicator of free-living physical activity. *Obes. Res.* 11, 865–868.

Westerterp, K. R., Meijer, G. A. L., Janssen, E. M. E., Saris, W. H. M., and Ten Hoor, F. (1992). Long term effect of physical activity on energy balance and body composition. *Br. J. Nutr.* 68, 21–30.

Westerterp, K. R., and Plasqui, G. (2009). Physically active lifestyle does not decrease the risk of fattening. *PLoS. ONE* 4:e4745. doi: 10.1371/journal.pone.0004745

Westerterp, K. R., Saris, W. H. M., Van Es, M., and Ten Hoor, F. (1986). Use of the doubly labeled water technique in man during heavy sustained exercise. *J. Appl. Physiol.* 61, 2162–2216.

Westerterp, K. R., and Speakman, J. R. (2008). Physical activity energy expenditure has not declined since the 1980s and matches energy expenditure of wild mammals. *Int. J. Obes.* 32, 1256–1263.

Westerterp, K. R., Verboeket-van de Venne, W. P. H. G., Bouten, C. V. C., De Graaf, C., Van het Hof, K. H., and Weststrate, J. A. (1996). Energy expenditure and physical activity in subjects consuming full- or reduced-fat diets. *Br. J. Nutr.* 76, 785–795.

Westerterp, K. R., Wouters, L., and Van Marken Lichtenbelt, W. D. (1995). The Maastricht protocol for the measurement of body composition and energy expenditure with labeled water. *Obes. Res.* 3(Suppl), 1, 49–57.

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 October 2012; paper pending published: 07 February 2013; accepted: 08 April 2013; published online: 26 April 2013.

*Citation: Westerterp KR (2013) Physical activity and physical activity induced energy expenditure in humans: measurement, determinants and effects. Front. Physiol. **4**:90. doi: 10.3389/fphys.2013.00090*

This article was submitted to Frontiers in Integrative Physiology, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

METHODS ARTICLE
published: 01 July 2013
doi: 10.3389/fphys.2013.00155

A standardized approach to study human variability in isometric thermogenesis during low-intensity physical activity

Delphine Sarafian, Jennifer L. Miles-Chan, Gayathri Yepuri, Jean-Pierre Montani, Yves Schutz and Abdul G. Dulloo*

Department of Medicine/Physiology, University of Fribourg, Fribourg, Switzerland

Edited by:
Patrick Christian Even, AgroParisTech, France

Reviewed by:
Patrick Christian Even, AgroParisTech, France
Klaas R. Westerterp, Maastricht University, Netherlands

****Correspondence:***
Abdul G. Dulloo, Department of Medicine/Physiology, University of Fribourg, Chemin du Musée 5, CH-1700 Fribourg, Switzerland
e-mail: abdul.dulloo@unifr.ch

Limitations of current methods: The assessment of human variability in various compartments of daily energy expenditure (EE) under standardized conditions is well defined at rest [as basal metabolic rate (BMR) and thermic effect of feeding (TEF)], and currently under validation for assessing the energy cost of low-intensity dynamic work. However, because physical activities of daily life consist of a combination of both dynamic and isometric work, there is also a need to develop standardized tests for assessing human variability in the energy cost of low-intensity isometric work.

Experimental objectives: Development of an approach to study human variability in isometric thermogenesis by incorporating a protocol of intermittent leg press exercise of varying low-intensity isometric loads with measurements of EE by indirect calorimetry.

Results: EE was measured in the seated position with the subject at rest or while intermittently pressing both legs against a press-platform at 5 low-intensity isometric loads (+5, +10, +15, +20, and +25 kg force), each consisting of a succession of 8 cycles of press (30 s) and rest (30 s). EE, integrated over each 8-min period of the intermittent leg press exercise, was found to increase linearly across the 5 isometric loads with a correlation coefficient (r) > 0.9 for each individual. The slope of this EE-Load relationship, which provides the energy cost of this standardized isometric exercise expressed per kg force applied intermittently (30 s in every min), was found to show good repeatability when assessed in subjects who repeated the same experimental protocol on 3 separate days: its low *intra*-individual coefficient of variation (CV) of $\sim 10\%$ contrasted with its much higher *inter*-individual CV of 35%; the latter being mass-independent but partly explained by height.

Conclusion: This standardized approach to study isometric thermogenesis opens up a new avenue for research in EE phenotyping and metabolic predisposition to obesity.

Keywords: obesity, spontaneous physical activity, NEAT, exercise, thermogenesis, energy expenditure, isometric, static

INTRODUCTION

The assessment of human energy expenditure (EE), under standardized conditions, has wide applications in human energy metabolism ranging from the estimation of energy requirements of population groups and individual hospitalized patients (Miles, 2006; Shephard and Aoyagi, 2012) to the elucidation of the genetic and metabolic basis of human susceptibility to obesity (Dulloo et al., 2012). In this context, standardized approaches for assessing human variability in EE measured at rest in the postabsorptive state as the basal metabolic rate (BMR) or in the postprandial state as the thermic effect of feeding (TEF) are well-defined (Schutz, 2008; Schutz and Dulloo, in press), as are measurements of the energy cost of moderate-to-high intensity exercise performed during treadmill walking/running, repetitive bench press and squat exercises or during cycling ergometry (Donovan and Brooks, 1977; Bijker et al., 2001; Robergs et al., 2007; Lazzer et al., 2011).

During the past decade, there has been considerable interest in the notion that EE associated with everyday life physical activities, often referred to as non-exercise activity thermogenesis, play an important role in the regulation of body weight (Dauncey, 1990; Levine et al., 2006; Garland et al., 2011). To study such low-intensity physical activities, however, is a challenging task as they include not only voluntary occupational and leisure activities but also subconscious spontaneous physical activity such as muscle tone and posture maintenance and fidgeting (Thompson et al., 2009; Westerterp, 2009). In addition to the development of accelerometers and activity monitors for the detection and quantification of these low-intensity activities (Wong et al., 2007; Melanson et al., 2009; Plasqui et al., 2013),

methodological approaches are currently being developed and validated for assessing EE variability in response to standardized dynamic exercise at low power outputs that are energetically comparable to low-intensity physical activities of daily life (Reger et al., 2012). However, because movements during daily life comprise not only dynamic work but also isometric (static) work, and that intermittent isometric thermogenesis is an important component of EE associated with spontaneous physical activity (Dulloo et al., 2012), there is a need to develop a standardized test for assessing human variability in the energy cost of intermittent isometric exercise of low-intensity.

To this end, we report here the development and validation of an approach that consists of incorporating a standardized protocol of intermittent leg press exercise of varying low-intensity isometric loads with measurements of EE by indirect calorimetry in a comfortable seated position. The specific objective of the study reported here was to test the feasibility and repeatability of this approach for assessing the energy cost of isometric thermogenesis in response to such intermittent low-intensity isometric work.

MATERIALS AND METHODS

CRITERIA FOR METHOD DEVELOPMENT

In the development of an appropriate methodological approach to study human variability in isometric thermogenesis pertaining to the field of nutrition and metabolism, several criteria were established. *First*, the standardized isometric exercise test should be feasible for incorporation with measurements of EE using indirect calorimetry by the ventilated-hood (canopy) system. *Second*, the isometric loads should be low in intensity and intermittent so as to mimic real life, with each isometric contraction alternating with a period of rest of similar duration, such that each cycle of isometric load and rest would lead to increases in EE that would be in the range of some of the low-level physical activities during everyday life, i.e., within 2-fold relative to resting levels. *Third*, the intermittent isometric loads should be low enough in intensity and short enough in duration so that min-by-min blood pressure is unlikely to increase more than marginally even at the highest intermittent isometric load. *Fourth*, the energy cost of the isometric exercise should be assessed by regression analysis of EE *vs* kg force loads applied, so as to enable the calculation of the "delta energy cost" of the exercise test (i.e., energy cost per kg force applied intermittently) by analogy to the calculation of delta mechanical efficiency for dynamic exercise.

STANDARDIZATION OF POSTURE AND ISOMETRIC LOADS

EE was measured by ventilated-hood indirect calorimetry (Deltatrac II, Datex-Ohmeda, Helsinki) in a comfortable seated position in an ergonomic and adjustable car seat which was mounted on a rectangular metal frame on wheels with strong brakes (**Figure 1A**). In order to apply the "hood" component of the indirect calorimetry system in the seated position, the top part of the seat's back-support was modified so as to incorporate a head-support (50 cm long × 50 cm wide × 1.5 cm thick) made of a wooden base covered with a sponge-filled cushion; the angle of inclination of the seat's back support was adjusted between 110 and 120°. The standardized posture of the subject at rest during baseline measurements was to sit in the car seat with the feet on the foot rest or along the sides of the metal frame. Isometric work was performed by pressing both legs simultaneously against a press-platform which consisted of an in-built metal frame carrying a weighing scale (Seca 862, Hamburg, Germany) that could tilt around its horizontal axis. The position of the press-platform, which could slide horizontally along the metal frame, is adjusted for each subject such that when the subject's feet are at rest and flat on the press-platform, the angle between subject's thigh (femur) and lower leg (tibia) is a right angle (90°). Under these conditions, the value obtained (without any active leg press) corresponded to the kg force exerted by the weight of the legs on the press-platform. This "passive leg load" was found to be highly reproducible both within and between days in a given subject, and to vary between 10 and 20 kg among the subjects participating in the experiment reported here. For each subject, this *passive* leg load is taken as a reference value which is then used to define 5 different *active* press load levels (i.e., 5 different isometric loads), namely +5, +10, +15, +20, and +25 kg force, and referred to as isometric load L1, L2, L3, L4, and L5, respectively.

DATA ACQUISITION AND VISUAL DISPLAY

The output of the weighing scale is relayed, through a digital monitor screen (Seca 862, Hamburg, Germany), to a laptop computer containing a custom-built software for data acquisition and continuous second-by-second screen display of the real-time kg force exerted on the press-platform (**Figure 1B**). Both the digital monitor screen and the computer laptop/screen are placed on a rack which is positioned approximately two meters in front of the subject. Prior to each exercise test, the set isometric load (kg force) that the subject should exert on the press-platform is introduced in the computer. During the leg press exercise, the actual isometric load exerted on the press-platform is continuously read by the computer software, and any deviation of 1 kg above or below the set isometric load triggers an electronic "beep" alarm which alerts the subject to maintain the leg press within ± 1 kg of the set load. The onset and termination of each press/rest cycle is signaled by the investigator.

DESIGN OF ISOMETRIC EXERCISE

Preliminary experiments were undertaken to establish the duration and number of press/rest cycles in line with the criteria set above. These criteria were met by performing a succession of 8 cycles of press/rest for each isometric load level, with the press (contraction) and rest (relaxation) periods lasting 30 s each. During the 30 s of rest within each cycle, the legs are kept "passively" on the press-platform (i.e. without exerting any active press). After each set of exercise bouts (i.e., after 8 press/rest cycles at a given isometric load level), the subject remains seated at rest for another 16 min, with the feet along the sides of the metal frame (as during the baseline period). The experimental design for a typical experimental run is presented schematically in **Figure 2**; the sequence of the 5 press loads applied during each experimental run for each subject is as follows: L1, L3, L5, L4, and L2.

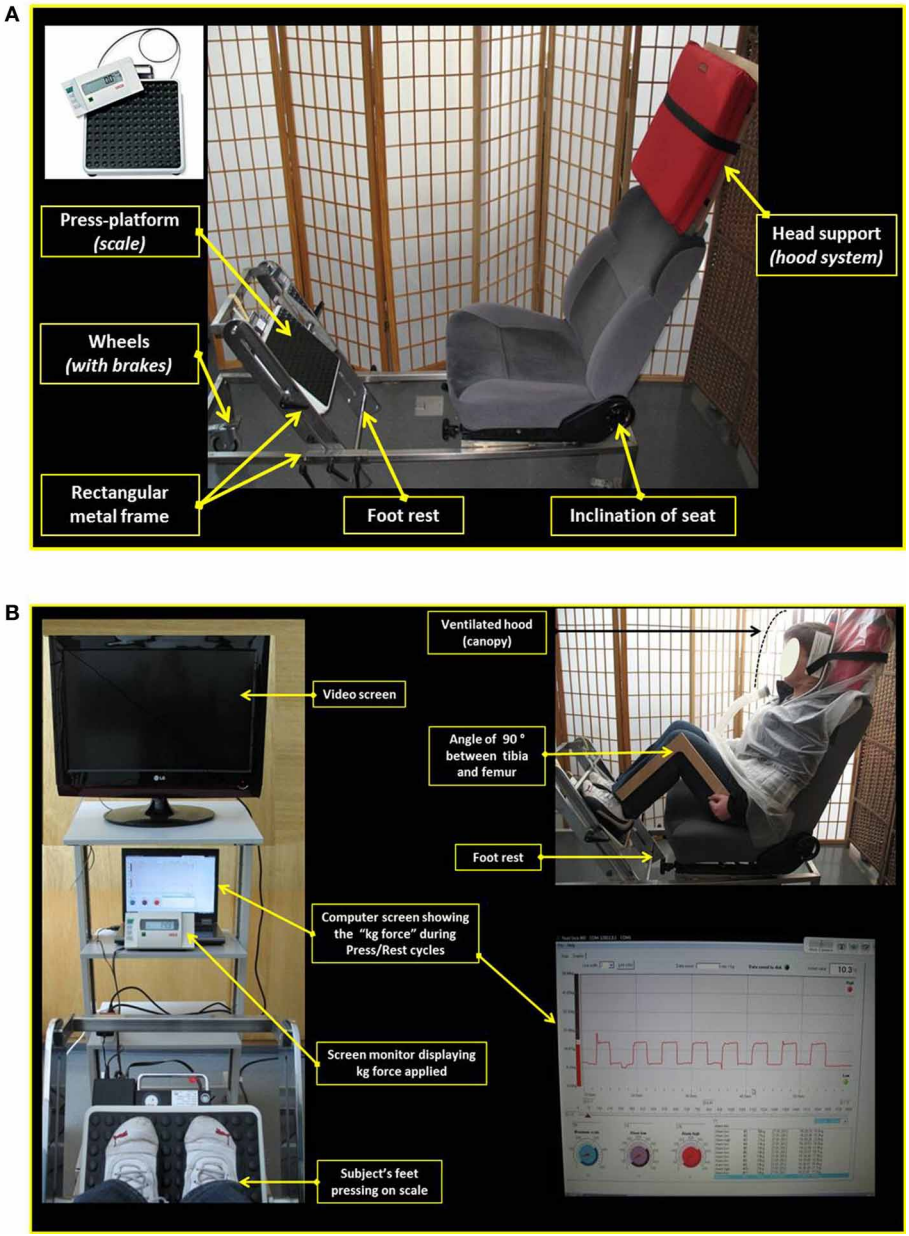


FIGURE 1 | (A) Picture showing the adapted car seat and the press-platform used for the standardized isometric exercise test (See text for details). **(B)** Standardized posture during isometric exercise (See text for details).

DATA ANALYSIS

An example of the EE profile in response to the five press loads in a young woman is shown as min-by-min values (kcal/min) in **Figure 3A**. At a given press load level, each EE data point is an integrated 1 min EE value; this corresponds to EE for one press/rest cycle, with each cycle consisting of 30 s of "press" and 30 s of "rest." Within the black circle which emphasizes the 8 min-by-min data points of EE in response to the 8 press/rest cycles at press load level 3 (L3), and amplified in **Figure 3B**, the data point labeled "1" corresponds to the EE in response to the 1st press/rest cycle, and so on, with the data point labeled "8" corresponding to the last minute EE data in response to the 8th press/rest cycle. The overall data of EE in response to each press load level—comprising the 8 press/rest cycles—is analyzed as the integrated mean EE of the 8 min EE data points corresponding to the 8 press/rest cycles; this is analogous to the method for calculating the energy cost (and delta efficiency) of dynamic work. For each run, a plot of EE *vs* isometric loads yields a strong linear relationship, with the correlation coefficient $r > 0.9$, as shown in **Figure 3C**. The slope of this linear regression therefore provides the energy cost of performing the standardized isometric exercise per kg force applied half of the time (i.e. per kg force $t_{1/2}$); this

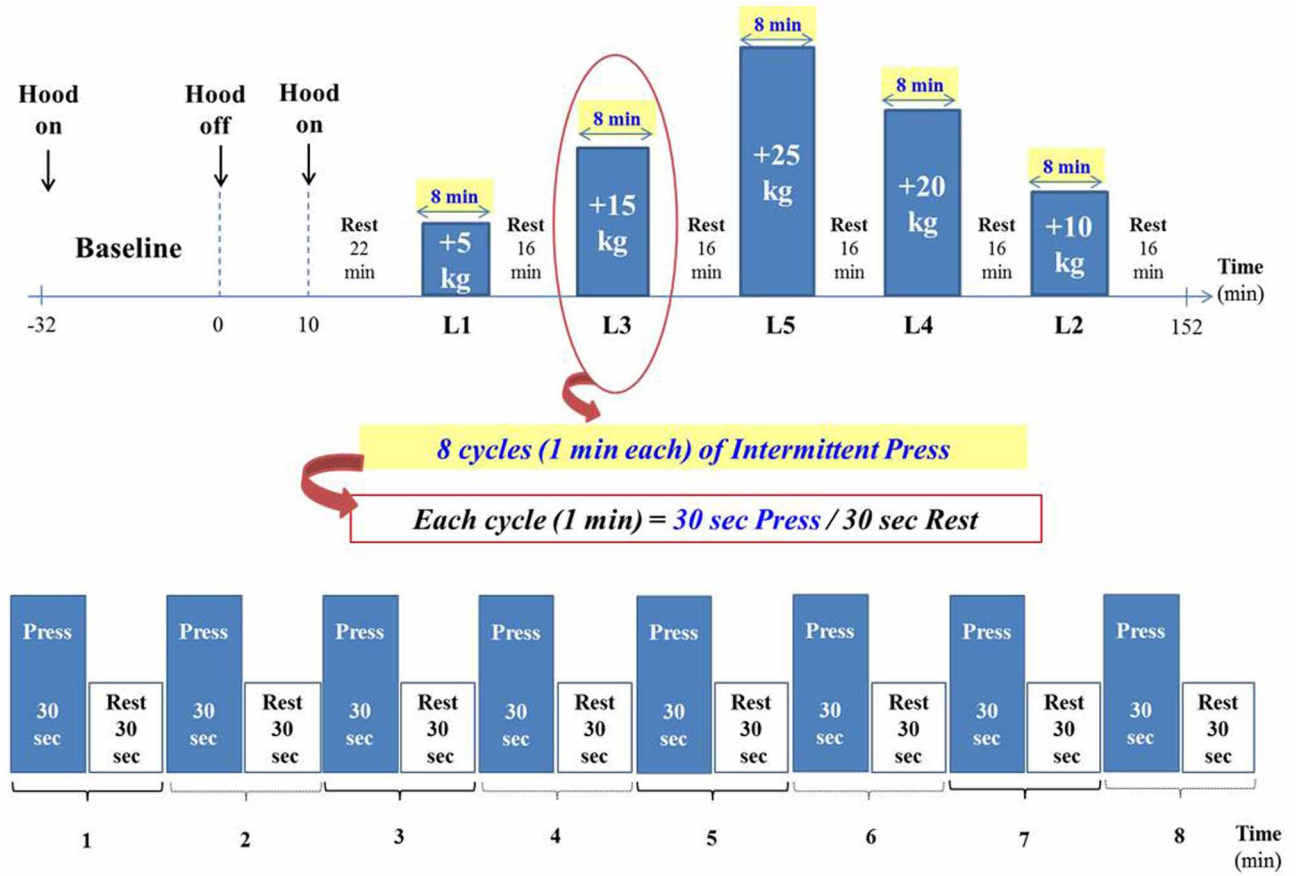


FIGURE 2 | Experimental design (See text for details). The duration of the entire exercise test is approximately 2.5 h, after 30 min of baseline measurements.

is referred to as the "delta energy cost" of the exercise, and can be calculated from regression lines that either exclude or include the baseline EE (BEE) value (i.e., EE at 0 isometric load), and expressed as kcal.min^{-1} / kg force $t_{1/2}$.

CARDIOVASCULAR MONITORING

Non-invasive cardiovascular recordings were performed using a Task Force Monitor (CNSystems, Medizintechnik, Graz, Austria) as described previously by Brown et al. (2008). Cardiac intervals (and their reciprocal, heart rate) were recorded by electrocardiography. Four ECG electrodes were placed on the subject's torso and thoracic impedance was recorded using three band electrodes, one placed on the back of the neck and two parallel electrodes placed on the lateral sides of the thorax at the level of the xiphoid process. Electrocardiogram and impedance cardiogram cables were then connected. Cardiac output and stroke volume were derived on a beat-to-beat basis from the impedance cardiogram (Kubicek et al., 1996). Continuous blood pressure was recorded during the baseline period and during the highest isometric load exercise (L5) from the index and middle fingers of the right hand alternatively using the vascular unloading technique, and calibrated to oscillometric brachial blood pressure measurements on the contralateral arm.

SUBJECTS AND REPEATABILITY EXPERIMENT

To assess the intra-individual variability in the energy cost of isometric exercise, the standardized leg press exercise at various isometric loads was conducted in 9 young non-obese adults (4 men and 5 women; age range of 21–29 years), with the test repeated in the same subject on 3 different days and with at least a two days interval between any 2 test days. All measurements were performed in the morning after an overnight fast, and after the determination of baseline EE at rest (BEE) over at least 30 min. None of the subjects were athletes or engaged in regular sports activities on daily or weekly basis. They all, however, considered themselves as being moderately active and fit, spending between 30–60 min walking during their daily activities. All subjects maintained a relatively stable body weight (within ± 1 kg) during the 3 months preceding the study. Exclusion criteria were as follows: regular smokers, claustrophobic subjects, pregnant or breastfeeding women, subjects with acute infections, chronic inflammatory disease or taking medication which could interfere with metabolic rate. Participants were asked to avoid intense physical activity and to abstain from caffeine-containing foods and beverages for 24 h before the test. Subjects were also required to eat their dinner before 20 h on the eve of the test, in order to comply with 12 h of fasting. All women were tested

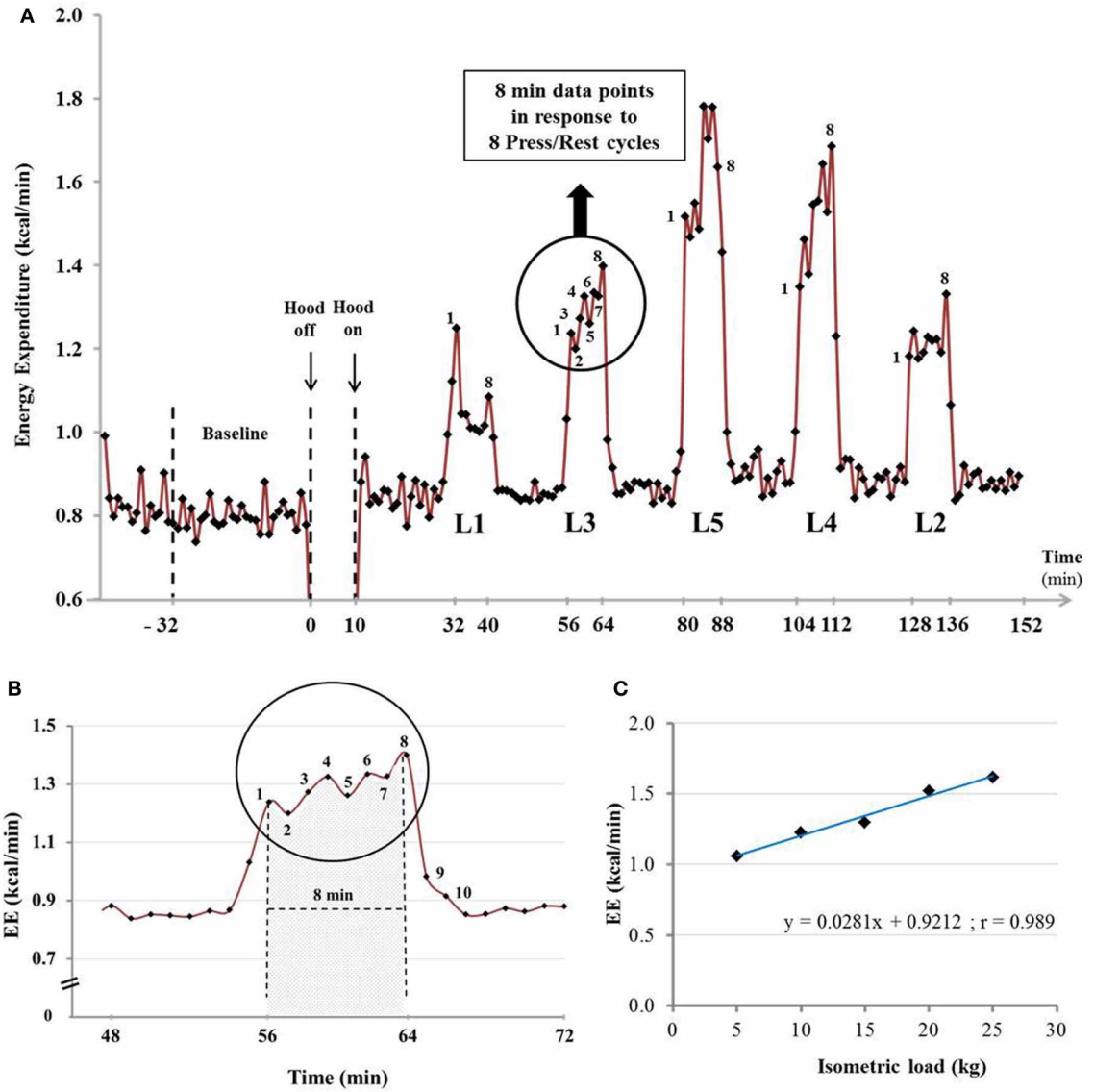


FIGURE 3 | (A) Example of the EE (kcal/min) profile in a young woman at rest (Baseline) and in response to the five isometric active loads (L1, L2, L3, L4, L5) recorded minute by minute by indirect calorimetry. The isometric (active) loads are: L1 = +5 kg, L2 = +10 kg, L3 = +15 kg, L4 = +20 kg and L5 = +25 kg. **(B)** Illustration of the computational method used for data analysis. The EE profile during isometric exercise at L3 (as shown above) is magnified. **(C)** Linear regression analysis on mean EE (kcal/min) data as a function of five isometric active loads (+5, +10, +15, +20, +25 kg). Note that this subject is not included in those who participated in the subsequent (repeatability) experiment.

in the follicular phase of their menstrual cycle. To minimize the effect of physical activity on the morning of the test day, participants were requested to use motorized transport instead of walking or cycling to reach the laboratory. Before enrollment in the study, the participants came to the laboratory for an interview where they completed a questionnaire about their lifestyle and medical history; anthropometry measurements (weight and height) were made using a mechanical scale with an integrated stadiometer (Seca model 709, Hamburg, Germany). On this occasion, the study objectives and procedures before and during the experiments were explained, and a written informed consent was obtained for all subjects. All procedures complied with the Declaration of Helsinki and received local ethics committee approval.

RESULTS

EE-LOAD RELATIONSHIP

The EE vs. isometric load regression line for each of the nine subjects on the first of their three separate test measurements is shown in **Figure 4**. Overall, EE is found to increase above baseline by 11–93% across the various loads L1–L5 (+5 to +25 kg force), with a strong linear EE-Load relation ($r > 0.95$) for each

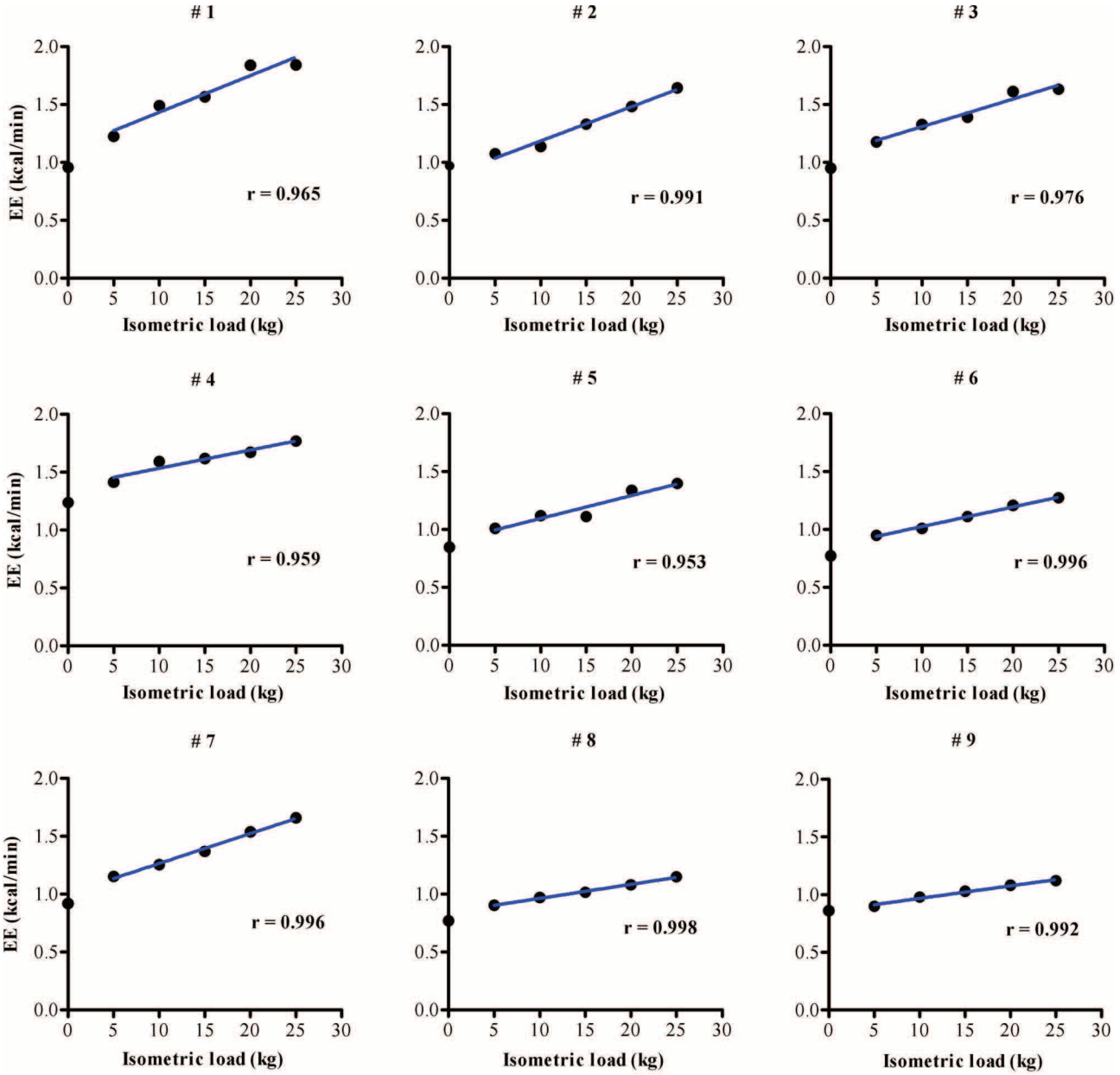


FIGURE 4 | Linear regression of EE (kcal/min) as a function of the five isometric active loads (+5, +10, +15, +20, +25 kg). The coefficient of regression (*r*) is shown for each subject. # denotes subject number.

subject. The distribution of the slope values, presented in ascending order in **Figure 5**, indicates a large inter-individual variability in the energy cost of performing this standardized exercise (>3-fold difference in slope values (range: 0.01–0.033). The value of the y-intercept (EE at zero isometric load) is found to be significantly higher ($p < 0.01$) than the measured BEE value by 0.063 kcal/min: an increase of EE above baseline of 6.7% on average, with 8 of 9 subjects showing y-intercept higher than BEE in the range of 3–12%. No significant differences were found between men and women in the values of y-intercept minus BEE, and in the slope values.

INTRA-INDIVIDUAL VARIABILITY IN EE

The data on coefficient of variation (CV%), based on three repeated measures on the three separate days for BEE and EE at each isometric load, as mean values for the nine subjects, are presented in **Figure 6**. As expected, across these 3 days, the intra-individual CV (intra-CV) of BEE, like that often reported for BMR (Adriaens et al., 2003), is within 5% with a mean intra-CV of 2.6% and range of 0.5–5%. At each press load (L1–L5), the mean intra-CV value for EE is also found to be <5%, with the individual intra-CV in the range of 1–8% across the various press loads. There are no significant differences in the mean intra-CV

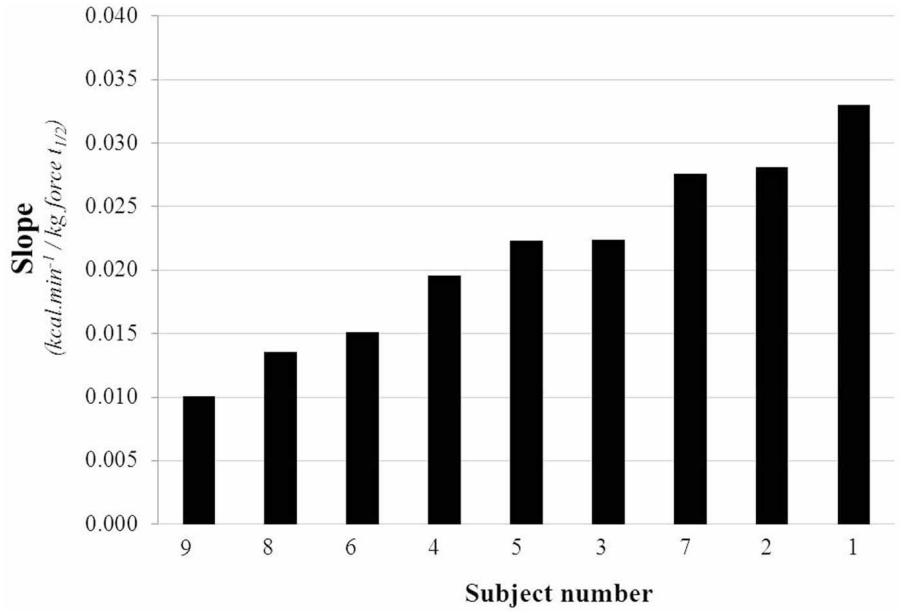


FIGURE 5 | Distribution of the slope values (3d-mean) assessed by linear regression on three separate days among the nine subjects.

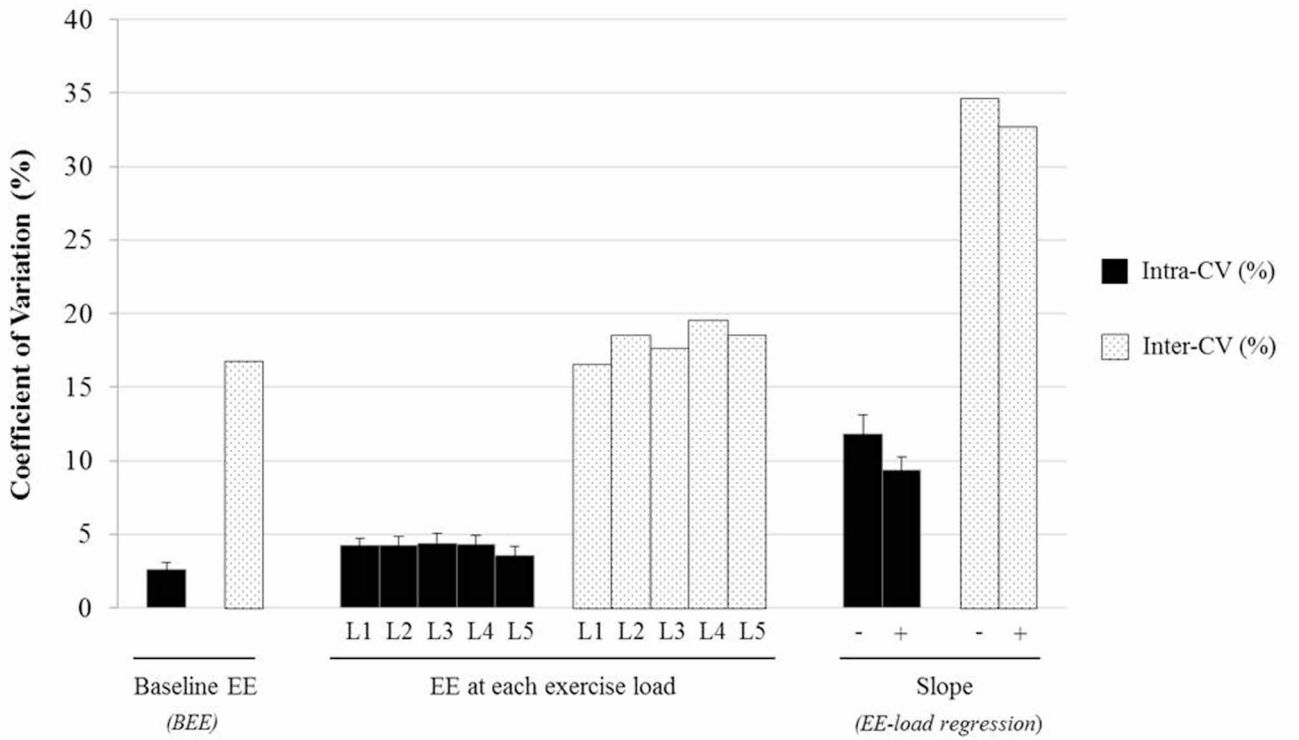


FIGURE 6 | Intra- and inter-individual coefficient of variation (CV%) of EE at rest and during isometric exercise in 9 young adults. The CV (%) was determined according to EE measured at baseline (BEE), EE at each active isometric load (L1, L2, L3, L4, L5) and for EE-Load regression slope measured on three separate occasions (3d-mean EE) with BEE excluded (−) or included (+) in the regression analysis. Values are Mean ± SEM for intra-CV and mean values for inter-CV.

for EE at each exercise load, with values for L1, L2, L3, L4, and L5 being within the narrow range of 3.6–4.4%. Furthermore, at any press load, the mean intra-CV (∼4%) is several times (4–5 folds) lower than the inter-individual CV (inter-CV) values which range between 16.6 and 19.6%.

INTRA-INDIVIDUAL VARIABILITY IN SLOPE OF EE-LOAD RELATIONSHIP

Comparison of the delta energy cost of the standardized exercise, assessed from the slope of the regression between EE and isometric loads, indicates that the large inter-individual variability among the nine subjects (mean inter-CV ∼ 35%) is 3 fold greater than the mean intra-CV value of 11.8% (range 6.4–17%) (**Figure 6**, right-hand columns). The inclusion of BEE (i.e., the measured EE at zero isometric load) in the regression analysis yielded slightly but significantly higher slope values (0.023 vs. 0.021, $p < 0.01$), as well as slightly but significantly lower mean intra-CV (9.3 vs. 11.8%, $p < 0.05$), with the mean inter-CV being 3.5 fold greater than the mean intra-CV, as shown in **Figure 6** (right-hand bars).

TRAINING/HABITUATION AND LOAD SEQUENCE EFFECTS

To test whether there is a systematic trend of changes in the slope values across the three measurement days, the ratio of the slopes of the EE-Load relationship were examined as follows: "Day 2 vs. Day 1," "Day 3 vs. Day 1," and "Day 3 vs. Day 2," and presented in **Figure 7**. The results indicate no systematic directional change in the slope values across the measurement days, thereby suggesting that there is no systematic training or habituation effect in the assessment of the energy cost of the exercise. Furthermore, the findings that the performance of the L4 and L2 loads after the highest load L5 (i.e., order sequence: L1, L3, L5, L4, L2) has no impact on the linear relationship between EE and isometric loads L1–L5 suggest that there are no time-dependent effects and no hysteresis in the observations.

OVERALL FINDINGS IN EE vs. LOAD RELATIONSHIP

This experiment above designed primarily to investigate the within-subject variability in the delta energy cost of the standardized isometric exercise suggests a much lower intra-individual variability compared to the inter-individual variability (intra-CV of ∼10% vs. inter-CV of 35%), and that the intra-CV in the delta energy cost of the standardized isometric exercise is not significantly different in men and women.

ANTHROPOMETRIC CORRELATES WITH EE IN RESPONSE TO PRESS LOADS

Analysis of anthropometric predictors of the large inter-individual variability in the energy cost of the isometric exercise at each load (total EE − BEE) as well as in the slope of the EE-Load regression show no correlation with body weight or with lower weight exponents (**Table 1**), as well as with the passive load (i.e., the kg force exerted by positioning the legs on the press-platform but without actively pressing); there was however a significant (or close to significant) association with height. This contrasts with baseline EE, which as expected, is found to be strongly correlated with both body weight and height.

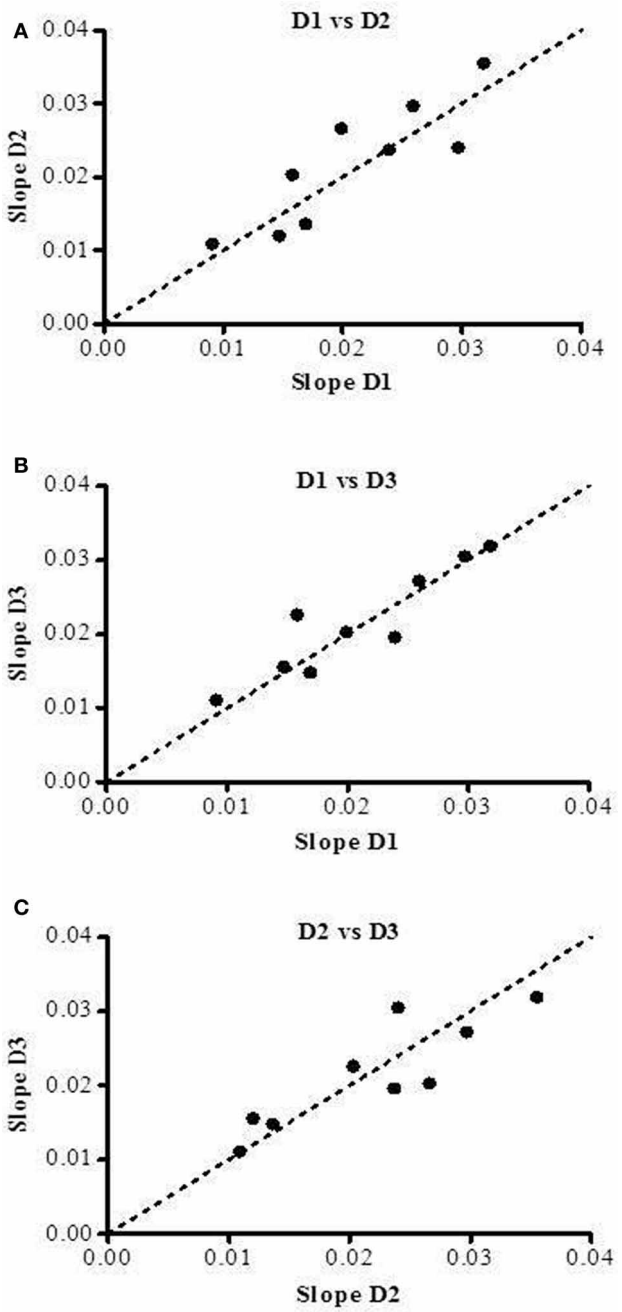


FIGURE 7 | Slope values obtained on the three separate days of testing (D1, D2, and D3) in nine subjects. Slope values of Day 2 and Day 3 are plotted against slope values of Day 1 (panels **A,B**) and slope values of Day 3 are plotted against slope values of Day 2 (panel **C**). The dotted line corresponds to the line of identity.

CARDIOVASCULAR RESPONSES

To validate that this approach to study the energy cost of isometric work does not lead to overt increases in total peripheral resistance (TPR) and blood pressure, as would be expected for isometric loads that are intermittent, of relatively short duration (30 s) and low in intensity (<30 kg force applied with the legs, and

Table 1 | Pearson's correlation analysis of baseline energy expenditure (EE) or BEE, activity energy expenditure (change in EE above baseline) and slope of EE-load regression vs anthropometry (body weight, lower body weight exponents, and height), as well as vs. the passive load (the kg force exerted by the weight of the legs on the press-platform).

		Baseline EE (BEE)	Slope (EE-load regression)	Change in EE above baseline				
				+5 kg	+10 kg	+15 kg	+20 kg	+25 kg
BODY WEIGHT (BW)								
BW^{1}	*r*	0.84	0.17	0.14	−0.03	0.16	0.02	0.18
	p	<0.01	NS	NS	NS	NS	NS	NS
$BW^{0.7}$	*r*	0.83	0.18	0.13	−0.03	0.16	0.02	0.19
	p	<0.01	NS	NS	NS	NS	NS	NS
$BW^{0.5}$	*r*	0.82	0.18	0.12	−0.03	0.16	0.02	0.19
	p	<0.01	NS	NS	NS	NS	NS	NS
$BW^{0.3}$	*r*	0.82	0.19	0.12	−0.03	0.15	0.02	0.19
	p	<0.01	NS	NS	NS	NS	NS	NS
$BW^{0.1}$	*r*	0.81	0.19	0.11	−0.03	0.15	0.03	0.20
	p	<0.01	NS	NS	NS	NS	NS	NS
Passive load	*r*	0.60	−0.03	−0.04	−0.21	−0.05	−0.18	−0.02
	p	=0.08	NS	NS	NS	NS	NS	NS
Height	*r*	0.79	0.70	0.71	0.54	0.77	0.63	0.76
	p	<0.01	<0.05	<0.05	=0.14	<0.05	=0.07	<0.05

NS, not statistically significant ($p > 0.05$); the p-values are also indicated for r-values close to statistical significance (p-value between 0.05 and 0.15).

<2-fold increase in EE above baseline), a comprehensive analysis of the cardiovascular response was monitored in subjects ($n = 7$) during baseline period at rest, during their highest press load, i.e., during the 8 press/rest cycles at press load L5, and during another 16 min after the press load exercise. A detailed 30 s. by 30 s. cardiovascular response to the intermittent isometric exercise for one individual (subject no. 8) is shown in **Figure 8**, and the results for all subjects as means of 16 min baseline followed by 8 min of intermittent exercise at highest press load (L5), and followed for another 16 min post-exercise are presented in **Figure 9**. In all these subjects, there were significant but small increases (<8 mm Hg) in systolic and diastolic blood pressure (**Figures 9A,B**) during the 8-min of intermittent exercise. Cardiac output was found to be significantly higher (+23%) and TPR to be significantly lower (−14%) during the exercise than at rest (panels E and F, respectively). The increase in cardiac output was characterized by an increase in both heart rate (panel C) and stroke volume (panel D).

RQ-LOAD RELATIONSHIP

Unlike for the EE-load relationship showing a robust linear regression of EE vs. isomertric loads applied, no significant relationship is found between RQ and the isometric loads (**Figure 10**).

DISCUSSION

The primary objective of this study was to extend the capacity for metabolic and EE phenotyping beyond those conducted in the resting state (BMR, TEF) or during dynamic exercise, by developing a standardized approach to study human variability in isometric thermogenesis in response to intermittent low-intensity isometric leg press exercise. In this methodological development, several considerations and criteria were taken into account in line with the long-term objective of applying this approach for investigations in the area of human nutrition and weight regulation. These are discussed below, first in relation to the practicality and feasibility of conducting the exercise protocol, and subsequently in relation to analytical issues in determining the energy cost of the isometric exercise.

SITTING POSTURE AND SEATED EXERCISE

As a first step in the development of the standardized exercise, it was important that the study was conducted with minimum discomfort for the subjects, while at the same time ensuring that the exercise test at different isometric loads would lead to increases in EE in a range compatible with low-intensity physical activity of everyday life. To this end, the subjects were seated in an ergonomic and adjustable car seat that was modified for enabling continuous assessment of EE with the subject at rest or while exerting intermittent leg press—an isometric exercise that would involve a large skeletal muscle mass. The seated position is also convenient for the subject to execute the isometric exercise as it allows easy reading of the visual feedback (Seca monitor) to check if the amount of force pressed on scale is close (±1 kg) to the pre-defined target value. Furthermore, in order to prevent boredom during the various rest periods between exercise bouts, the

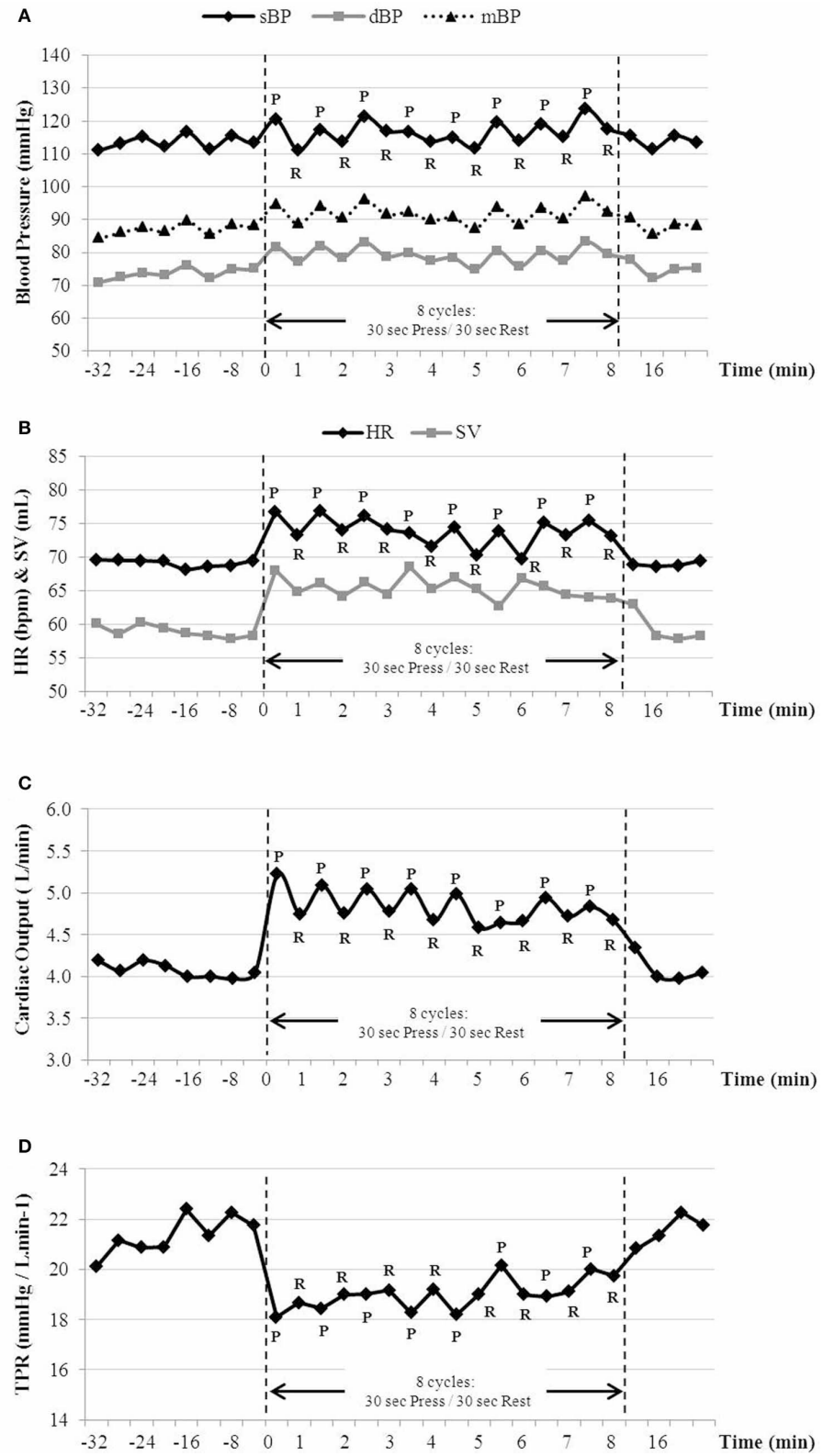


FIGURE 8 | Cardiovascular parameters in response to the highest isometric load (*L5* = +25 kg) in one subject (#8). For the 8 press/rest cycles performed at the highest load, **(A)** blood pressure (BP), **(B)** heart rate (HR), stroke volume (SV), **(C)** cardiac output and **(D)** total peripheral resistance (TPR) were averaged as 30 s during leg press (P) and rest (R). Each data point before and after pressing at the highest load represents rest periods and were processed as 4 min means.

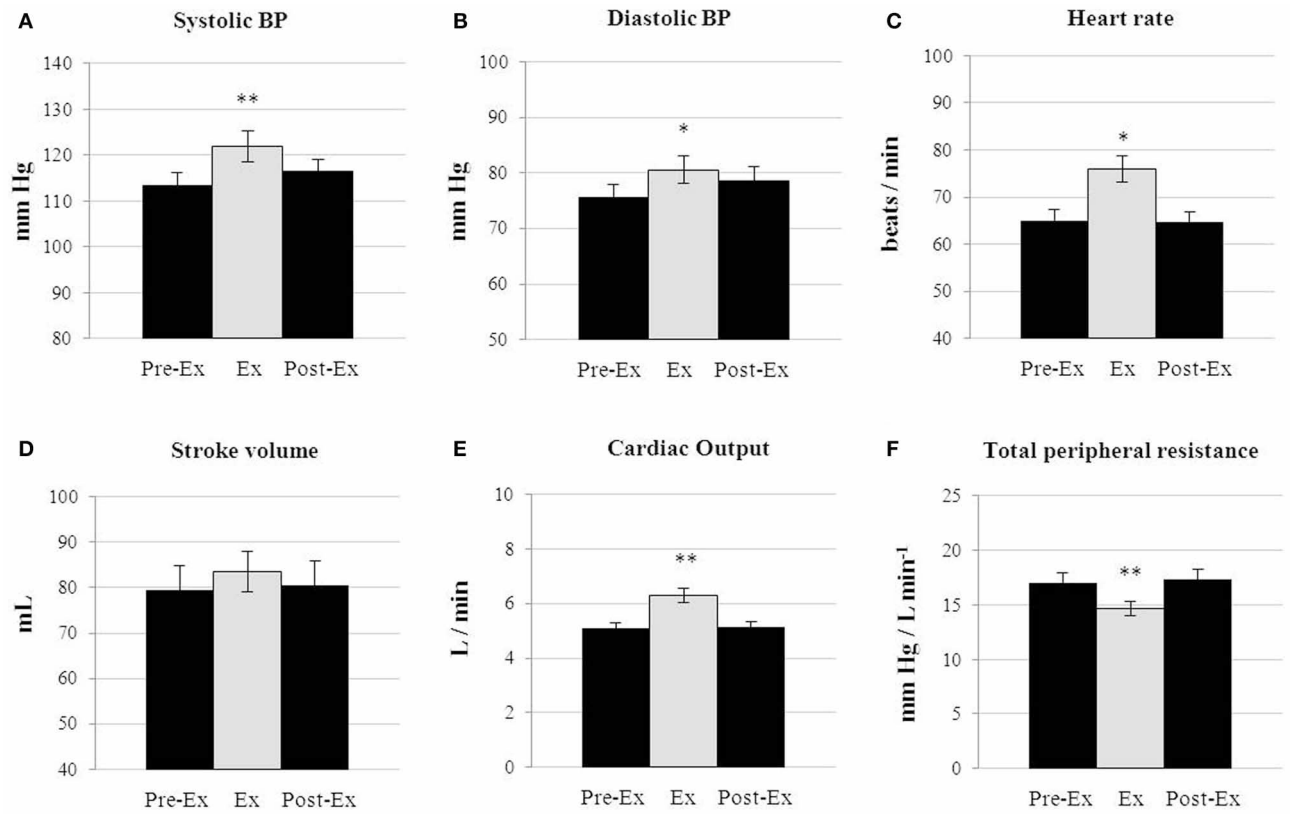


FIGURE 9 | Effect of the intermittent isometric exercise on hemodynamics. Cardiovascular parameters (blood pressure, heart rate, stroke volume, cardiac output and total peripheral resistance) in response to the highest press load (+25 kg force) in subjects ($n = 7$), and compared to the rest periods before (Pre-Ex Rest) and after (Post-Ex Rest) the highest press load (Ex). Statistical significance of differences is indicated with the symbol ∗ denoting statistical significance as assessed by ANOVA repeated measures; $^{*}p < 0.05$, $^{**}p < 0.01$.

seated position provides a suitable posture for watching a documentary/film on a video screen placed on a rack in front of the subject. Overall, the entire protocol, which involves continuous monitoring of EE by indirect calorimetry during the various periods of rest and intermittent leg press exercise, was found to be simple and easy to perform by the subjects, and feasible to be co-ordinated by the investigator.

VENTILATED-HOOD CALORIMETRY

Breath-to-breath gas analysis by an indirect calorimetry system with the subject wearing an appropriate facemask or mouthpiece/nose-clip is generally applied to monitor EE during exercise. Although it is not feasible to monitor high rates of EE of several METs by the ventilated-hood technique—due to non-adjustable air flow rate limitations and CO_2 build-up—this system can nonetheless be used to assess much more modest increases in EE such as during our study involving low-intensity and intermittent leg press exercise where EE is below 2 METs even at the highest press load. Indeed, in our study EE was found to increase linearly above baseline by 17–62% on average across the 5 loads levels, with the lowest individual value at L1 (+5 kg) and highest individual value at L5 (+25 kg) corresponding to increases in the range of 11–93%. This range is comparable to increases in EE above supine levels observed during standing motionless (+13%) (Levine et al., 2000), sitting while typing (+50%) (Ainsworth et al., 2011) and during standing while fidgeting (Levine et al., 2000) or during cycling against zero watt (no-load cycling) (+70 to 90%) (Reger et al., 2012), and is clearly below the 3–4 fold increase in EE observed during walking on a treadmill at 3–5 km/h (Levine et al., 2000). The incorporation of a head-support to the back of the seat allowed EE to be measured by the ventilated-hood indirect calorimetry system in the seated position. The fact that the exercise test involves only the lower part of the body (i.e., both legs) makes the measurement easy to perform with the components of the ventilated-hood system (hood and plastic veil) covering upper body while sitting at ease. The advantage of the ventilated-hood system is that it allows the measurement of gas exchange from an individual's "natural" breathing, which is often reported to be more comfortable than when using a facemask or mouthpiece with nose-clip; these tending to be more stressful, leading to overestimation of EE. Indeed, EE at rest in subjects wearing a mouthpiece/nose-clip system or face-mask system have been found to be 6-8% higher than when measured by the ventilated hood system (Forse, 1993; Roffey et al., 2006). Moreover, the use of the ventilated-hood system is more suitable for

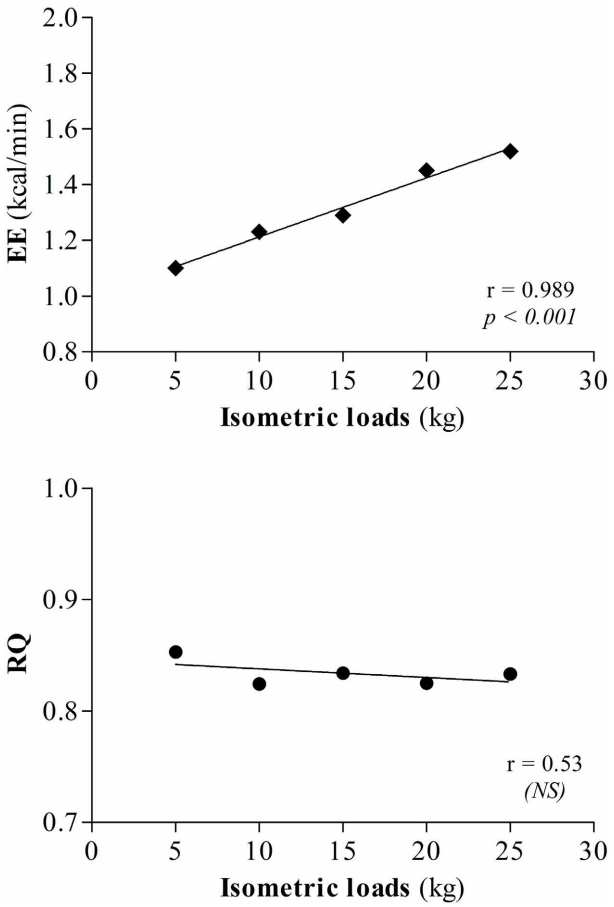


FIGURE 10 | Linear regression of EE (kcal/min) or respiratory quotient (RQ) as a function of the five isometric active loads (+5, +10, +15, +20, +25 kg).

studies that are of long duration (several hours) as in our test where several periods of rest are needed after each isometric load level.

BYPASSING REDUCED MUSCLE BLOOD FLOW LIMITATIONS

An assumption of our study is that the intermittent low-intensity contractions of 1 min cycles of 30 s press/30 s rest would not result in any sustained reduction in blood flow and hence would not induce increased lactate formation as has been demonstrated when static contraction is sustained for several minutes or until exhaustion (Cerretelli et al., 1976; Gorostiaga et al., 2010). Whilst we did not measure muscle lactate formation or blood lactate concentrations, our findings showed that the min-by-min TPR was reduced (by 14% on average) during the highest isometric load applied, suggesting that min-by-min blood flow actually increased during intermittent isometric exercise under conditions of our study. Indeed, the significant reduction in TPR counteracted much of the expected large increase in blood pressure classically seen in sustained isometric exercise (Friedman et al., 1992) leading to only small increases (<8 mm Hg) in blood pressure even at the highest intermittent isometric load. Since isometric contractions were alternated with equal time periods of rest in our standardized test, the diminution in TPR observed in our results can be explained by similar phenomenon to that occurring in dynamic exercise where TPR decreases due to vasodilation of the arterioles, allowing more blood and oxygen into the working muscle. Furthermore, compared to an intermittent static exercise performed with the arms (handgrip tests), the larger muscle mass recruited in our task (both legs) would induce a greater vasodilatation and might explain the observed decrease in TPR.

CHOICE OF ISOMETRIC LOAD STANDARDIZATION

By comparison with standardized dynamic exercise (i.e., using a cycle ergometer or treadmill) where the intensity of physical activity is generally expressed as a percentage of VO_2 max and the power output measured in watts, most studies related to isometric exercise have defined the intensity of workloads as a percentage of maximal voluntary contraction (MVC). However, depending on both physical and mental status (co-operation, motivation) of the subject and the type/nature of the isometric exercise, the determination of the MVC during isometric tests can be highly variable within the same individual; i.e., it has a poor reproducibility. In preliminary experiments in our laboratory on four healthy young men aimed at assessing within-subject variability in maximum leg press isometric contraction performed in our car seat set-up, we found CV% as high as 40–70% for MVC (data not shown); these latter findings led us to abandon the use of percentage MVC in the standardization of the exercise load in our study. The use of a percentage of body weight instead of a percentage of MVC has been suggested to be a more appropriate measure of load level (Kondraske et al., 1987), but as our study was conducted in the seated position, and hence the body weight mostly supported by the seat, it seems more relevant that the isometric load to be exerted on the press-platform was determined as a function of weight of the subject's leg on the press-platform. The "passive" leg load, which ranged between 10 and 20 kg in our subjects, was used to define the 5 press load levels during our exercise test by adding to each subject's passive leg load, the five different active loads (+5, +10, +15, +20, +25 kg). The sequence of isometric loads was fixed for all participants and consisted of a mixture of ascending and descending intensity force level during the exercise test in order to avoid any systematic ordering effects. Under these conditions, the strong linear relationship between EE and the isometric loads applied intermittently ($r > 0.9$ for all subjects) provides an appropriate analytical approach for determining the energy cost of the standardized exercise by linear regression and to phenotype individuals for thermogenesis in response to intermittent isometric work, and hence in variability in isometric thermogenesis.

LINEARITY OF EE-LOAD RELATIONSHIP

Because this analytical approach—based upon EE-Load linear regression—is analogous to the calculation of delta efficiency in dynamic exercise, it is referred to here as the "delta energy cost" of the isometric exercise. It is to be noted that amidst decade old debates regarding the ideal efficiency calculation for dynamic exercise, the calculation of delta efficiency is believed to most

closely approximate muscle efficiency (Gaesser and Brooks, 1975; Poole and Henson, 1988; Ettema and Loras, 2009). Furthermore, as recently emphasized by Reger et al. (2012) in referring to the delta efficiency for dynamic exercise, "*the slope may be the best indicator of exercise efficiency since the slope reflects the metabolic cost of biological processes that increase as power outputs increase*." In the field of Physics, however, the term "efficiency" does not apply to isometric work since the definition of physical work (Joule) implies a force (Newton) and displacement (meter). But in isometric or static work, there is no muscle shortening (displacement)—as in our task where both legs of the participant are pushing against a fixed, stationary platform without moving it through a distance; in other words, according to the physics definition, no external work is performed on the environment. Consequently, in our study focused on measurements of EE during isometric exercise, the strong EE-Load linear regression observed allows the calculation of the delta energy cost of the standardized isometric exercise (expressed per kg force applied intermittently).

RELIABILITY IN ASSESSING THE ENERGY COST OF ISOMETRIC EXERCISE

In developing a new approach to study EE, it is important to investigate the extent to which the approach is reliable, that is to test its repeatability and stability under the different conditions in which it is likely to be used. In this context, it is important to emphasize that variability in measurements of EE encompasses multiple errors that can derive from the instrument, the investigator and the biological variability of the subject, i.e., within-individual variability, day-to-day variability (Donahoo et al., 2004). The contribution of the instrument and investigator error to measures of EE can be minimized by performing (as done in this study), routine maintenance of equipment and the calibration of the calorimeter before each test and using the same measuring equipment for each test: within-instrument errors of <2% and between-machine errors <3% have been reported for EE measured by the Deltatrac (Phang et al., 1990; Wells and Fuller, 1998), the indirect calorimetry system utilized in our studies. As for the contribution of biological variability to the overall error, this was minimized by having a standardized protocol for every subject to follow, all measurements were conducted after overnight fast, and women were all assessed in the follicular phase of their menstrual cycle. Under these conditions, our experiment in nine subjects who repeated the standardized isometric exercise protocol on three separate days reveals a good repeatability for EE at rest and during each exercise level as judged by a mean intra-individual CV of 2.6% for BEE, 4% for EE at each of the five isometric loads applied, and 12% for the slope of the EE-Load relationship, i.e. for the delta energy cost of the isometric exercise. The higher intra-CV obtained for the delta energy cost of the exercise than for EE at each press load (12 vs. 4%) may be explained by the fact that unlike the analysis of each load separately, the regression slope reflects the energy cost through the range of 5 isometric loads (grouped variables). From a mathematical standpoint, the CV of a slope obtained by regression analysis is not comparable to the CV obtained on absolute values: it is more sensitive to small changes in both the numerator and denominator. However, the assessment of the energy cost of the exercise per kg force across a range of loads is a more accurate determination of this parameter than its assessment based upon only 1 or 2 loads.

EE AT REST: MEASURED VS PREDICTED FROM THE EE-LOAD RELATIONSHIP

A consistent finding in the EE-Load relationship is that the y-intercept of this linear regression is significantly higher than for baseline EE at rest (BEE), indicating the metabolic cost of biological processes that remain constant across the various exercise loads. One explanation may be that the EE-Load relationship is not linear, but curvilinear, across the range of very low loads in the range of 0–5 kg force. Alternatively, the higher value of the y-intercept than the BEE value could be reflecting an increased EE associated with the psychobiological conscious "act" of performing the various exercise bouts, increased intra-abdominal pressure, or increased muscle tension in the abdomen/trunk during these types of exercise.

APPLICATION OF THE STANDARDIZED TEST IN THE POSTPRANDIAL STATE

This approach developed here for studying isometric thermogenesis can also be applied to investigations in the postprandial state, although there are two prerequisites for this application, namely that the EE-Load relationship should be assessed in a phase of relative steady-state of postprandial EE, and that the time taken to reach this steady-state after the meal ingestion is not too long (say < 1 h). This approach nonetheless underscores the feasibility of its application for investigations into the impact of nutrient composition on the delta energy cost of isometric exercise in the postprandial state.

ANTHROPOMETRIC CORRELATES AND PERSPECTIVE

In line with the main purpose of this study, the criterion to perform intermittent isometric exercise of low intensity with EE comparable with low-level activities of everyday life is met in our study as judged by the findings that EE increases linearly above baseline by 17–62 % on average across the five loads levels (+5 to +25 kg); with the lowest individual value at L1 (+5 kg) and highest individual value at L5 (+25 kg) corresponding to increases in the range of 11–93%. Of particular importance in the methodological development for the assessment of the delta energy cost of isometric exercise is the fact that the inter-CV is several fold greater than the intra-CV, with the slope values ranging from 0.01 to 0.03 (kcal.min^{-1})/kg force $t_{1/2}$. This raises the pertinent question for future research in a large population sample about what could be the determinants of such large inter-individual variability in the energy cost of the standardized isometric exercise (i.e., variability in isometric thermogenesis)?

From an analysis of anthropometric predictors of this large variability in the energy cost of the isometric work, it is shown here (**Table 1**) that unlike the expected high correlation between baseline EE (at rest) and body weight, there is no significant correlation between the activity EE and body weight expressed to various weight exponents, including those proposed by Prentice et al. (1996) for normalizing various activities for differences in body

weight; there was also no correlation with the passive load, i.e., the kg force exerted by positioning the legs on the press-platform but without actively pressing. By contrast, there is a significant correlation with height, suggesting that linear dimensions, but not weight, is a predictor of the large inter-individual variability in isometric thermogenesis in response to the standardized exercise described here. The results of these correlation analyses must, however, be regarded as preliminary and treated with great caution given that it is based on a small sample size ($n = 9$), which while appropriate for methodological validation of feasibility and repeatability, clearly lacks power for providing predictors of inter-individual variability. Large scale studies would be required to confirm the magnitude of the inter-individual variability in isometric thermogenesis, its independency of body weight (and various weight exponents), and to address the importance of stature, the components of stature (lower vs. upper body length), regional body composition and objectively measured fitness in explaining human variability in isometric thermogenesis.

In the meantime, it can be speculated that as this standardized isometric exercise involves specifically the legs in the sitting position, it is most likely that it is leg length rather than height *per se* that explain part of the variability in isometric thermogenesis. Given reports from large epidemiological studies for an association between adult short stature (and/or short leg length) and increased risks for cardiovascular diseases (Paajanen et al., 2010), type 2 diabetes (Asao et al., 2006) and obesity (Hermanussen et al., 2005; Bosy-Westphal et al., 2009), it is tempting to put forward the hypothesis here that low isometric thermogenesis could constitute a metabolic link in the inverse association between stature/leg length and cardiometabolic risks. According to this hypothesis, individuals that have lower stature/leg length would tend to have a lower energy cost for the same workload, and hence spend less energy when performing the same isometric work than individuals with higher stature/leg length. Such energy sparing may constitute a thrifty metabolism link that predisposes people with genetically or environmentally-induced short stature/leg length to increased risks for obesity and cardiovascular diseases.

CONCLUSIONS

This standardized approach to study isometric thermogenesis (in response to intermittent low-intensity isometric workloads), as a complementary approach for EE phenotyping at rest or during dynamic activity, opens up a wide avenue for research in EE and metabolic phenotyping, with implications for research in human energy metabolism, and potential for a better understanding of metabolic predisposition to obesity.

ACKNOWLEDGMENTS

The authors would like to thank André Gaillard for various construction and adaptations that enabled integration of seated posture, leg press system, and ventilated hood indirect calorimetry, Laurent Monney for building the in-house software, Bernard Aebischer for the electronics, and Dr. Clive Brown for useful discussions in the early stage of the method development.

REFERENCES

Adriaens, M. P., Schoffelen, P. F., and Westerterp, K. R. (2003). Intra-individual variation of basal metabolic rate and the influence of daily habitual physical activity before testing. *Br. J. Nutr.* 90, 419–423. doi: 10.1079/BJN2003895

Ainsworth, B. E., Haskell, W. L., Herrmann, S. D., Meckes, N., Bassett, D. R., Tudor-Locke, C., et al. (2011). Compendium of physical activities: a second update of codes and MET values. *Med. Sci. Sports Exerc.* 43, 1575–1581. doi: 10.1249/MSS.0b013e31821ece12

Asao, K., Kao, W. H., Baptiste-Roberts, K., Bandeen-Roche, K., Erlinger, T. P., and Brancati, F. L. (2006). Short stature and the risk of adiposity, insulin resistance, and type 2 diabetes in middle age: the third national health and nutrition examination survey (NHANES III), 1988–1994. *Diabetes Care* 29, 1632–1637. doi: 10.2337/dc05-1997

Bijker, K. E., De Groot, G., and Hollander, A. P. (2001). Delta efficiencies of running and cycling. *Med. Sci. Sports Exerc.* 33, 1546–1551. doi: 10.1097/00005768-200109000-00019

Bosy-Westphal, A., Plachta-Danielzik, S., Dörhöfer, R. P., and Müller, M. J. (2009). Short stature and obesity: positive association in adults but inverse association in children and adolescents. *Br. J. Nutr.* 102, 453–461. doi: 10.1017/S0007114508190304

Brown, C. M., Dulloo, A. G., Yepuri, G., and Montani, J. P. (2008). Fructose ingestion acutely elevates blood pressure in healthy young humans. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 294, R730–R737.

Cerretelli, P., Veicsteinas, A., Fumagalli, M., and Dell'orto, L. (1976). Energetics of isometric exercise in man. *J. Appl. Physiol.* 41, 136–141.

Dauncey, M. J. (1990). Activity and energy expenditure. *Can. J. Physiol. Pharmacol.* 68, 17–27. doi: 10.1139/y90-002

Donahoo, W. T., Levine, J. A., and Melanson, E. L. (2004). Variability in energy expenditure and its components. *Curr. Opin. Clin. Nutr. Metab. Care* 7, 599–605. doi: 10.1097/00075197-200411000-00003

Donovan, C. M., and Brooks, G. A. (1977). Muscular efficiency during steady-rate exercise. II. Effects of walking speed and work rate. *J. Appl. Physiol.* 43, 431–439.

Dulloo, A. G., Jacquet, J., Montani, J. P., and Schutz, Y. (2012). Adaptive thermogenesis in human body weight regulation: more of a concept than a measurable entity. *Obes. Rev.* 13, 105–121.

Ettema, G., and Loras, H. W. (2009). Efficiency in cycling: a review. *Eur. J. Appl. Physiol.* 106, 1–14. doi: 10.1007/s00421-009-1008-7

Forse, R. A. (1993). Comparison of gas exchange measurements with a mouthpiece, face mask, and ventilated canopy. *JPEN J. Parenter. Enteral Nutr.* 17, 388–391. doi: 10.1177/0148607193017004388

Friedman, D. B., Peel, C., and Mitchell, J. H. (1992). Cardiovascular responses to voluntary and nonvoluntary static exercise in humans. *J. Appl. Physiol.* 73, 1982–1985.

Gaesser, G. A., and Brooks, G. A. (1975). Muscular efficiency during steady-rate exercise: effects of speed and work rate. *J. Appl. Physiol.* 38, 1132–1139.

Garland, T. Jr., Schutz, H., Chappell, M. A., Keeney, B. K., Meek, T. H., Copes, L. E., et al. (2011). The biological control of voluntary exercise, spontaneous physical activity and daily energy expenditure in relation to obesity: human and rodent perspectives. *J. Exp. Biol.* 214, 206–229. doi: 10.1242/jeb.048397

Gorostiaga, E. M., Navarro-Amézqueta, I., Cusso, R., Hellsten, Y., Calbet, J. A., Guerrero, M., et al. (2010). Anaerobic energy expenditure and mechanical efficiency during exhaustive leg press exercise. *PLoS ONE* 5:10. doi: 10.1371/journal.pone.0013486

Hermanussen, M., Sunder, M., Voigt, M., and Tresguerres, J. A. (2005). Morbid obesity is associated with short stature. *J. Pediatr. Endocrinol. Metab.*18, 647–650.

Kondraske, G. V., Deivanayagam, S., Carmichael, T., Mayer, T. G., and Mooney, V. (1987). Myoelectric spectral analysis and strategies for quantifying trunk muscular fatigue. *Arch. Phys. Med. Rehabil.* 68, 103–110.

Kubicek, W. G., Karnegis, J. N., Patterson, R. P., Witsoe, D. A., and Mattson, R. H. (1996). Development and evaluation of an impedance cardiac output system. *Aerosp. Med.* 37, 1208–1212.

Lazzer, S., Plaino, L., and Antonutto, G. (2011). The energetics of cycling on earth, moon and mars. *Eur. J.*

Appl. Physiol. 111, 357–366. doi: 10.1007/s00421-010-1410-1

Levine, J. A., Schleusner, S. J., and Jensen, M. D. (2000). Energy expenditure of nonexercise activity. *Am. J. Clin. Nutr.* 72, 1451–1454.

Levine, J. A., Vander Weg, M. W., Hill, J. O., and Klesges, R. C. (2006). Non-exercise activity thermogenesis: the crouching tiger hidden dragon of societal weight gain. *Arterioscler. Thromb. Vasc. Biol.* 26, 729–736. doi: 10.1161/01.ATV.0000205848.83210.73

Melanson, E. L., Dykstra, J. C., and Szuminsky, N. (2009). A novel approach for measuring energy expenditure in free-living humans. *Conf. Proc. IEEE Eng. Med. Biol. Soc.* 2009, 6873–6877. doi: 10.1109/IEMBS.2009.5333124

Miles, J. M. (2006). Energy expenditure in hospitalized patients: implications for nutritional support. *Mayo Clin. Proc.* 81, 809–816. doi: 10.4065/81.6.809

Paajanen, T. A., Oksala, N. K., Kuukasjärvi, P., and Karhunen, P. J. (2010). Short stature is associated with coronary heart disease: a systematic review of the literature and a meta-analysis. *Eur. Heart J.* 31, 1802–1809. doi: 10.1093/eurheartj/ehq155

Phang, P. T., Rich, T., and Ronco, J. (1990). A validation and comparison study of two metabolic monitors. *JPEN. J. Parenter. Enteral Nutr.* 14, 259–261. doi: 10.1177/0148607190014003259

Plasqui, G., Bonomi, A. G., and Westerterp, K. R. (2013). Daily physical activity assessment with accelerometers: new insights and validation studies. *Obes. Rev.* 14, 451–462.

Poole, D. C., and Henson, L. C. (1988). Effect of acute caloric restriction on work efficiency. *Am. J. Clin. Nutr.* 47, 15–18.

Prentice, A. M., Goldberg, G. R., Murgatroyd, P. R., and Cole, T. J. (1996). Physical activity and obesity: problems in correcting expenditure for body size. *Int. J. Obes.* 20, 688–691.

Reger, M., Peterman, J. E., Kram, R., and Byrnes, W. C. (2012). Exercise efficiency of low power output cycling. *Scand. J. Med. Sci. Sports* doi: 10.1111/j.160008382012.01448.x. [Epub ahead of print].

Robergs, R. A., Gordon, T., Reynolds, J., and Walker, T. B. (2007). Energy expenditure during bench press and squat exercises. *J. Strength Cond. Res.* 21, 123–130. doi: 10.1519/00124278-200702000-00023

Roffey, D. M., Byrne, N. M., and Hills, A. P. (2006). Day-to-day variance in measurement of resting metabolic rate using ventilated-hood and mouthpiece and nose-clip indirect calorimetry systems. *JPEN J. Parenter. Enteral Nutr.* 30, 426–432. doi: 10.1177/0148607106030005426

Schutz, Y. (2008). "Physical activity and the thermic effect of food," in *Advances in Physical Activity and Obesity*, eds C. Bouchard and P. T. Katzmarzyk (Champaign, IL: Human kinetics), 132–136.

Schutz, Y., and Dulloo, A. G. (in press). "Resting metabolic rate, thermic effect of food and obesity," in *Handbook of Obesity: Etiology and Pathophysiolog,* eds G. A. Bray and C. Bouchard (New York, NY: Marcel Dekker Inc.).

Shephard, R. J., and Aoyagi, Y. (2012). Measurement of human energy expenditure, with particular reference to field studies: an historical perspective. *Eur. J. Appl. Physiol.* 112, 2785–2815. doi: 10.1007/s00421-011-2268-6

Thompson, D., Batterham, A. M., Markovitch, D., Dixon, N. C., Lund, A. J., and Walhin, J. P. (2009). Confusion and conflict in assessing the physical activity status of middle-aged men. *PLoS ONE* 4:2. doi: 10.1371/journal.pone.0004337

Wells, J. C., and Fuller, N. J. (1998). Precision and accuracy in a metabolic monitor for indirect calorimetry. *Eur. J. Clin. Nutr.* 52, 536–540. doi: 10.1038/sj.ejcn.1600604

Westerterp, K. R. (2009). Assessment of physical activity: a critical appraisal. *Eur. J. Appl. Physiol.* 105, 823–828.

Wong, W. Y., Wong, M. S., and Lo, K. H. (2007). Clinical applications of sensors for human posture and movement analysis: a review. *Prosthet. Orthot. Int.* 31, 62–75

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 May 2013; accepted: 10 June 2013; published online: 01 July 2013.
Citation: Sarafian D, Miles-Chan JL, Yepuri G, Montani J-P, Schutz Y and Dulloo AG (2013) A standardized approach to study human variability in isometric thermogenesis during low-intensity physical activity. Front. Physiol. 4:155. doi: 10.3389/fphys.2013.00155
This article was submitted to Frontiers in Integrative Physiology, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 04 June 2013
doi: 10.3389/fphys.2013.00128

Assessment of brown adipose tissue function

Sam Virtue*[1] *and* ***Antonio Vidal-Puig*[1,2]**

[1] *Metabolic Research Laboratories, Addenbrooke's Treatment Centre, Institute of Metabolic Science, Addenbrooke's Hospital, University of Cambridge, Cambridge, UK*
[2] *Wellcome Trust Sanger Institute, Cambridge, UK*

Edited by:
Patrick Christian Even, AgroParisTech, France

Reviewed by:
Kazuhiro Nakamura, Kyoto University, Japan
Jan Nedergaard, Stockholm University, Sweden

****Correspondence:***
Sam Virtue, Metabolic Research Laboratories, Addenbrooke's Treatment Centre, Institute of Metabolic Science, Addenbrooke's Hospital, University of Cambridge, Level 4, Box 289, Hills Road, Cambridge CB2 0QQ, UK
e-mail: sv234@medschl.cam.ac.uk

In this review we discuss practical considerations for the assessment of brown adipose tissue in rodent models, focusing on mice. The central aim of the review is to provide a critical appraisal of the utility of specialized techniques for assessing brown adipose tissue function *in vivo*. We cover several of the most common specialized methods for analysing brown adipose tissue function *in vivo*, including assessment of maximal thermogenic capacity by indirect calorimetry and the measurement of sympathetic tone to brown adipose tissue. While these techniques are powerful, they are not readily available to all laboratories; therefore we also cover several simple measurements that, particularly in combination, can be used to determine if a mouse model is likely to have alterations in brown adipose tissue function. Such techniques include: pair feeding, analysis of brown adipose tissue lipid content and mRNA and protein markers of brown adipose tissue activation.

Keywords: brown adipose tissue, BAT, maximal thermogenic capacity, adipose tissue, energy expenditure, cold exposure

INTRODUCTION

The aim of this review is to discuss the practical considerations of current methods for assessing brown adipose tissue (BAT) function in rodents, focusing largely on mice. While it is impossible to ignore the theoretical concepts behind assessment of BAT, these were recently covered in a comprehensive review (Cannon and Nedergaard, 2011).

BAT is a thermogenic organ and it acts to generate heat in order to maintain thermal homeostasis. As will be discussed below, nutrient oxidation in BAT can account for over 60% of the total energy expenditure of the mouse. Given the enormous effect of BAT on metabolic rate, alterations in BAT activity can impact on multiple different metabolic variables and therefore understanding the effect of BAT is essential for any branch of murine metabolic phenotyping.

THE IMPACT OF COLD EXPOSURE ON DIFFERENT METABOLIC PARAMETERS

Mice housed at 5°C exhibit metabolic rates two and half times higher than mice acclimated to 30°C. This dramatic increase in metabolic rate can impact on multiple aspects of metabolism. Cold exposure can render mice resistant to diet induced obesity (Cannon and Nedergaard, 2009). Additionally, the rate of insulin-independent glucose disposal in cold acclimated mice is so high it can prevent the manifestation of hyperglycaemia in streptozotocin treated rats, a type 1 diabetic model (Takano et al., 1987). Remarkably, there is at least one case report of systemic administration of thyroid hormone, which leads to BAT activation, being able to overcome the effects of a partial loss of function mutation in the insulin receptor (type A insulin resistance) in humans (Skarulis et al., 2010). In addition to its impacts on carbohydrate metabolism, cold exposure can: (1) affect nervous control of the cardiovascular system (Swoap et al., 2008), (2) confound the interpretation of activity data from calorimetric studies (Virtue et al., 2012a), and (3) normalize serum triglyceride levels in Apoa5-null mice (Bartelt et al., 2011). Given that 60% of the calories consumed by wild-type, cold-acclimated mice are oxidized in BAT, the potential for BAT to mediate cold-induced changes in carbohydrate and lipid metabolism is substantial and must be considered when interpreting data regarding whole-organism metabolic changes.

HOW DOES BAT GENERATE HEAT?

In order to understand how cold exposure and BAT activity can have such a dramatic impact on all aspects of metabolism it is necessary to understand the function of BAT. BAT is a thermogenic organ and its principal function is to convert nutrients to heat. The production of heat by BAT is also called non-shivering thermogenesis (NST). In order to generate heat, BAT possesses a unique protein called uncoupling protein 1 (UCP1). UCP1 acts to uncouple oxidative phosphorylation from ATP production. While its exact molecular mechanism is still a topic of debate, UCP1 allows protons to pass from the mitochondrial intermembrane space into the mitochondrial matrix. Activated UCP1 therefore sets up a futile cycle where the electron transport chain (ETC) pumps protons across the inner mitochondrial membrane and UCP1 allows them to flow back into the mitochondrial matrix. Crucially, UCP1 activity dissipates the inner mitochondrial membrane potential, which normally acts to limit the ETC. Therefore, the theoretical potential for BAT to oxidize nutrients is limited only by its nutrient supply and capacity for oxidative metabolism and, in line with this fact, BAT has the highest metabolic rate of any organ.

The term "BAT" is in some regards a relatively arbitrary description. Almost all adipose tissue depots in the mouse contain a mixture of brown and white adipocytes (Murano et al., 2009). Importantly, the relative balance of brown and white adipocytes can be dramatically increased or decreased by environmental and pharmacological interventions.

In recent years, it has become apparent that brown adipocytes located in different depots are derived from different populations of precursors. Brown adipocytes located in canonical BAT depots are suggested to come from a precursor that is common to brown adipocytes and muscle (Timmons et al., 2007; Seale et al., 2008). Conversely, brown adipocytes located in predominantly white adipose tissue (WAT) depots are suggested to share a precursor with white adipocytes. Brown adipocytes found in WAT depots have distinct transcriptional profiles from canonical brown adipocytes and have therefore been classed as a separate cell type, known as brite or beige cells, to reflect their intermediate profile between canonical brown and white adipocytes.

IMPACT OF TEMPERATURE ON METABOLIC RATE

The specific generation of heat may at first glance seem a relatively simple function for an organ, particularly given that heat is a by-product of most metabolic processes. However, virtually all aspects of BAT have evolved to maximize its capacity to oxidize nutrients. In an animal housed at 5°C, 60% of all the energy expended by a mouse is done so in BAT (Golozoubova et al., 2004; Cannon and Nedergaard, 2011). To allow such high oxidative rates BAT requires a substantial blood supply in order to provide nutrients and oxygen and to carry away waste products and heat. To that end, cold exposure causes extensive angiogenesis within BAT (Xue et al., 2009). In addition to specific changes in BAT in response to cold exposure, metabolic changes necessary for the mouse to adapt to cold exposure occur in multiple other organs. Animals increase their heart weight and increase their cardiac output to provide more blood flow in order to supply nutrients to either BAT (for NST) or muscle for shivering (Shechtman et al., 1990).

In contrast to cold exposure, heat production by BAT falls to almost nothing at thermoneutrality. The thermoneutral zone is defined as a temperature at which an organism does not have to employ active heat production nor evaporative heat dissipation to maintain its core body temperature. In mice the thermoneutral zone falls between 28°C and 33°C dependent on strain, gender and age. Below the thermoneutral zone an organism must expend energy to generate heat. This may be generated through processes such as shivering in muscle or NST, believed to occur principally in BAT. Above the thermoneutral zone animals must use energy to actively reduce their body temperature. Processes such as sweating, panting or saliva spreading on fur can promote heat loss to a lesser or greater extent, dependent on the organism.

Below the thermoneutral zone, in a C57Bl/6 mouse, energy expenditure increases by 8% per 1°C drop in environmental temperature (relative to energy expenditure at thermoneutrality) (Virtue et al., 2012a). Thus, a mouse expending 0.33 W at 30°C will expend 0.66 W at 18°C and 1 W at 5°C. The increase in energy expenditure in response to a reduction in environmental temperature appears to be linear, at least as far as 5°C. This has important implications when considering "room temperature" experiments in mice. Standard laboratory conditions range from 20 to 24°C, therefore a 30 g mouse housed at 24°C could be expected to expend approximately 0.5 W, whereas at 20°C it would be expected to expend 0.6 W. It is worth noting that 0.1 W is a 20% difference in metabolic rate—a difference larger than the impact of most genetic manipulations. Therefore, it is critical when considering any metabolic study to both control the environmental temperature and to state it as accurately as possible to allow reproduction of results.

THE SYMPATHETIC NERVOUS SYSTEM

Importantly, BAT activity is able to rapidly adapt to changes in environmental temperature. A sudden shift in environmental temperature from 5 to 30°C is met by a very rapid and proportionate reduction in energy expenditure in order to prevent hyperthermia. Unsurprisingly, these rapid adaptations are under nervous control. The principal arm of the nervous system that regulates BAT activity is the sympathetic nervous system (SNS).

The SNS is the single most important regulator of BAT function (Cannon and Nedergaard, 2004). The SNS regulates both acute BAT function and also prolonged BAT adaptation. The principal adrenergic receptor responsible for activating mature brown adipocytes is the β3-adrenergic receptor. The β3-adrenergic receptor also promotes differentiation of brown adipocytes that have been cultured for 6 days (Rehnmark et al., 1990; Bronnikov et al., 1999). On brown preadipocytes that have been cultured for up to 3 days the β1-adrenergic receptor appears to be the most prominent receptor and mediates noradrenergic stimulation of brown adipocyte proliferation (Bronnikov et al., 1992, 1999; Cannon and Nedergaard, 2004).

Activation of the β3-adrenergic receptor on mature brown adipocytes causes a rise in intracellular cyclic adenosine monophosphate (cAMP). This in turn activates lipolysis via protein kinase A (PKA)-mediated phosphorylation of the lipid droplet-associated protein perilipin (Souza et al., 2007). Phosphorylation of perilipin releases the protein Comparative Gene Identification-58 (CGI-58) which in turn activates adipose triglyceride lipase (ATGL) (Granneman et al., 2007, 2009). ATGL predominantly catalyses the break down of triglyceride to diglyceride (Zimmermann et al., 2004). Elevated lipolysis increases intracellular free fatty acid (FFA) levels, which activate UCP1. The absence of ATGL renders mice profoundly cold-intolerant, either due to insufficient FFAs for UCP1 activation, or due to a failure to mobilize FFAs from lipid droplets within BAT and WAT to provide substrates for fatty acid oxidation (Haemmerle et al., 2006). In addition to ATGL, hormone sensitive lipase (HSL) is also activated by PKA-mediated phosphorylation (Miyoshi et al., 2007). HSL appears to be less critical for thermal homeostasis than ATGL, with mice lacking HSL exhibiting normal cold tolerance (Osuga et al., 2000). However, HSL is directly phosphorylated in response to β-adrenergic stimulation by PKA on a number of serine residues (Holm, 2003), making HSL phosphorylation a useful molecular marker of BAT activation. When sympathetic tone to BAT falls, lipolysis diminishes and FFA levels fall, reducing UCP1 activation. Purinergic nucleotides can subsequently bind to UCP1

and inactivate it (Nicholls, 1974), allowing a rapid switching off of BAT thermogenesis.

In addition to the stimulatory role of the β3-adrenergic receptor, the α2-adrenergic receptor acts to inhibit the response of BAT in the presence of norepinephrine. The α2-adrenergic receptor is G_i coupled, and its stimulation lowers intracellular cAMP levels. Inhibition of α2-adrenergic receptors actually increases cAMP levels and the expression of UCP1 in brown adipocytes when they are stimulated with norepinephrine (Bronnikov et al., 1999).

ACUTE vs. ADAPTED COLD EXPOSURE

BAT undergoes extensive remodeling in response to cold, with this remodeling taking 3–5 weeks in mice. Cold exposure leads to a pronounced increase in BAT mass via adipogenesis and an increase in mitochondrial density. Total UCP1 protein mass increases in BAT during acclimation to the cold and overall thermogenic capacity is increased (Nedergaard and Cannon, 2013). Cold acclimation also promotes BAT angiogenesis (Xue et al., 2009) and also increases adipose tissue sympathetic nerve fiber density (Murano et al., 2009). Therefore, when studying mice with alterations in BAT function, it is important to consider at which stage of thermogenic adaptation an animal was when interpreting results from metabolic experiments. Warm or cold **exposure** is usually used to indicate an acute change in temperature lasting up to perhaps 72 h. Importantly, during cold exposure, BAT will be in the process of dynamic remodeling to either increase or decrease its thermogenic capacity. Conversely, cold or warm **acclimation** usually refers to a period of time of between 7 days and 3 months of housing at a constant temperature. After acclimation, BAT is assumed to have reached a steady state with respect to its level of thermogenic capacity. It is important to note that BAT will not be fully cold acclimated until between 3 and 5 weeks after moving from a room temperature (20–24°C) to a cold environment (5°C), therefore in some "cold acclimation" studies, BAT may still be undergoing remodeling.

A further complexity comes from the fact that reports investigating the impact of short-term exposure to either cold or warm environments often use different prior acclimation temperatures, which will affect the thermogenic capacity of mice. Additionally, studies also expose mice to different final temperatures, which will also affect the degree of BAT activation.

In general the majority of published literature regarding both exposure and acclimation experiments focuses on four major temperature ranges; thermoneutrality (28–33°C) when BAT activity is assumed to be negligible; "standard laboratory housing conditions" (20–24°C); cold exposure (usually 4–5°C), probably chosen as it is the temperature most commercial refrigerators maintain; and 18°C, in part used as an intermediate temperature chosen because (1) some mouse strains will not tolerate the transfer from 30 to 5°C, and (2) because it allows the detection of intermediate phenotypes that cannot be distinguished by shifts from 30 to 24°C or 30°C to 5°C (Golozoubova et al., 2001).

Overall, when considering any study, an appreciation of the environmental temperature an experimental animal was housed at prior to the experiment commencing and, if different, the temperature the study was conducted at is essential for interpretation of the results.

SHIVERING vs. NON-SHIVERING THERMOGENESIS (NST)

Full cold acclimation takes between 3 and 5 weeks dependent on mouse strain and magnitude of the thermal challenge (Nedergaard and Cannon, 2013). When a mouse is transferred from environments above 18°C to the cold (5°C), the thermogenic capacity of BAT is initially insufficient to maintain core body temperature, so heat must be generated from other sources. The principal acute source of thermogenesis in the mouse is shivering. Electromyography (EMG) traces show strong elevations in nervous tone to muscle when mice are first exposed to cold (Golozoubova et al., 2001). Over the first 4 weeks of cold exposure, EMG readings fall as BAT takes over heat generation from muscle. After 1 month of cold exposure, cold acclimated mice have similar EMG readings to mice that are housed at thermoneutrality, indicating that BAT is fully capable of maintaining thermal homeostasis. Notably, the UCP1 KO mouse, which cannot generate heat in BAT, maintains an elevated EMG reading even after 1 month of cold acclimation, supporting the concept that these mice principally use shivering thermogenesis to generate heat, even in a chronic setting (Golozoubova et al., 2001).

COMMON FACTORS THAT INDUCE BAT ACTIVITY

DIET

The concept of luxuskonsumption, now called diet-induced thermogenesis (DIT), was first identified in the nineteenth century based on the observation that over eating in both humans and dogs was associated with lower rates of weight gain than expected given the increase in caloric intake. In the 1970s Rothwell and Stock discovered that DIT in rats was associated with a series of metabolic changes in BAT, consistent with increased thermogenesis (Rothwell and Stock, 1979, 1983). Feldman et al. have demonstrated that UCP1 KO mice housed at thermoneutrality have greater weight gain on a high-fat diet than wild-type mice (Feldmann et al., 2009). This result provides the first conclusive evidence that BAT is a site of DIT. An important unresolved question is how high-fat feeding leads to the activation of adaptive thermogenesis.

TEMPERATURE

As already mentioned, temperature is the single biggest factor affecting BAT function and overall metabolic rate. For mice housed below thermoneutrality, the increase in energy expenditure per degree centigrade decrease in environmental temperature has been estimated to be between 6% (Herrington, 1940) and 8% (Virtue et al., 2012a).

DRUGS

A large number of drugs have been shown to modify energy expenditure and alter BAT function. The compounds that most directly affect BAT activity are those targeting the β-adrenergic system. Direct activation of β3-adrenergic receptors by agents such as CL316243 increases energy expenditure. In addition to their effects on energy expenditure β3-adrenergic agonists also promote brown adipocyte

differentiation (Cannon and Nedergaard, 2004). Additionally, the thiazolidinedione class of drugs target the nuclear hormone receptor peroxisome proliferator-activated receptor gamma (PPARγ) and promote the browning of WAT in rodents (Sell et al., 2004). A second critical nuclear hormone receptor for BAT development is the thyroid hormone receptor (Silva, 2006). Mice lacking the enzyme deiodinase 2 (Dio2), which is necessary for the conversion of T4 to the active T3 form in BAT, have impaired cold tolerance. Furthermore, lack of Dio2 was associated with reduced lipolysis and oxygen consumption in isolated brown adipocytes (de Jesus et al., 2001). Thyroid hormone replacement therapy in humans has also been associated with browning of WAT (Skarulis et al., 2010). Drugs that modulate the SNS, including cocaine, amphetamines (Kong et al., 2003) and ephedrine (Baba et al., 2007), also have known thermogenic actions. Serotonergic drugs can increase BAT activity by promoting the release of catecholamines from adrenergic neurons in BAT (Steiner and Evans, 1976). Nicotine has also been demonstrated to exert some of its weight reducing effects through activation of BAT (Martínez de Morentin et al., 2012). Overall, when using any drug which has weight-lowering actions, a potential effect on BAT should be considered and/or assessed.

INSULATION

Insulation is perhaps one of the single most important variables when considering energy balance. A lack of comprehension regarding the impacts of skin barrier dysfunction or hair-loss can lead to the misinterpretation of results. Perhaps the best example of how skin barrier function can affect metabolic results is the Stearoyl-CoA desaturase 1 (SCD1) KO mouse. Much of the phenotyping of the SCD1 KO mouse focused on the fact that SCD1 KO mice were resistant to diet induced obesity and had improved insulin sensitivity. Importantly, the SCD1 KO mouse has a dramatic loss of fur, leading to very poor thermal insulation. Consequently at any given temperature below thermoneutrality, the SCD1 KO mouse is under a much greater cold stress than a wild-type mouse (Ntambi et al., 2002). In order to meet the greater thermal challenge, SCD1 mice have to generate more heat than wild-type controls and as a result are hyper-metabolic (Binczek et al., 2007). As mentioned above, elevated metabolic rate is able to protect against many metabolic disorders, including diet-induced obesity. Of note, mice which only lack SCD1 in liver and thus have normal skin barrier function were not resistant to high-fat diet-induced obesity, but still exhibited improved insulin sensitivity (Miyazaki et al., 2007).

CALORIMETRY—A POWERFUL TOOL FOR MEASURING BAT FUNCTION

The principal product of BAT is heat. As such, calorimetry represents probably the single most useful tool for assessment of BAT function *in vivo*. Indirect calorimetry by gas exchange is the most common form of calorimetry used to measure energy expenditure in rodents. Gas-exchange indirect calorimetry relies on calculating energy expenditure from the consumption of oxygen (O_2) and production of carbon dioxide (CO_2) by an organism.

CALCULATING ENERGY EXPENDITURE FROM OXYGEN CONSUMPTION AND CO_2 PRODUCTION

Energy expenditure (EE) can be calculated from the amount of O_2 consumed (called VO_2) and the amount of CO_2 produced (VCO_2) using the Weir equation (Equation 1). The amount of energy produced per mole of oxygen consumed varies dependent on substrate by approximately 6%, with fatty acid oxidation producing less energy per mole of oxygen than carbohydrate oxidation. Therefore, when assessing metabolic rate it is necessary to calculate energy expenditure rather than just relying on VO_2, particularly when there are changes in substrate utilization (for example when switching from a high carbohydrate diet to a high-fat diet, or when analysing mice that are undergoing fasting, where endogenous lipid stores are oxidized).

$$\mathrm{EE}(J) = 15.818\mathrm{VO}_2 + 5.176\mathrm{VCO}_2 \quad (1)$$

A critical aspect of energy expenditure calculations is that VO_2 and VCO_2 are not equal under most conditions. The oxidation of different macronutrients results in the production of different quantities of CO_2. The ratio VCO2/VO2 is known as the respiratory exchange ratio (RER) or respiratory quotient (RQ) and can provide information about substrate oxidation. The RERs for the three major macronutrients are 1 for carbohydrate, 0.7 for lipid and approximately 0.9 for protein, dependent on amino acid composition. If considering RER only in terms of nutrient oxidation, then RER would be expected to range from 1 (pure carbohydrate oxidation) to 0.7 (pure lipid oxidation).

INTERCONVERSION OF NUTRIENTS AND RESPIRATORY EXCHANGE RATIO

While RER predominantly provides information about nutrient oxidation, several other metabolic processes can impact on the RER of an organism, leading to RER values which exceed the ranges possible purely from the oxidation of nutrients. In practice RER values are often observed which exceed 1, particularly when mice are fed a diet high in carbohydrate (such as laboratory chow diets which usually have a carbohydrate content of 60% or more). The process of *de novo* lipogenesis, by which carbohydrate is converted to lipid, has an RER of approximately 5. While *de novo lipogenesis* will only ever account for a small amount of all CO_2 production, it is not uncommon to observe RER values of 1.1 during periods when mice are synthesizing lipid.

A second point regarding RER is that it can be affected by changes in energy balance. Animals which are losing fat mass will have lower RERs than if they are weight-stable. When in negative energy balance, mice will rapidly utilize all their glycogen and begin oxidizing fat, which will reduce their RER relative to a weight-stable animals as oxidizing fat has an RER of 0.7. When considering differences between two groups of animals (for example a group of wild-type mice and a group of knock-out mice) it is important to consider any changes in body weight that occur between the groups while they are being measured. If one group of animals has lost more weight than the other they will almost certainly exhibit a reduction in RER, however, this reduction may be secondary to a change in energy balance, as opposed to a primary effect of altered lipid or carbohydrate handling.

IMPACT OF BAT ON THE METABOLIC RATE OF FREE-LIVING ANIMALS

As mentioned previously, metabolic rate is greatly increased by reductions in ambient temperature. Following full cold acclimation, heat production in wild-type mice largely occurs in BAT, therefore any differences in cold induced energy expenditure compared to controls is indicative of altered BAT function. However, it is important to realize that an increase in metabolic rate *per se* does not necessarily indicate an increase in BAT activity. UCP1 KO mice lack the ability to generate heat in BAT by uncoupled respiration, and predominantly use muscle shivering to maintain core body temperature (Golozoubova et al., 2001, 2006). Loss of BAT function, however, does not reduce total daily energy expenditure, with UCP1 KO mice having been demonstrated to have a metabolic rate at least as high as wild-type mice in multiple studies (Inokuma et al., 2006; Ukropec et al., 2006; Meyer et al., 2010). Therefore, an assessment of metabolic rate in response to changes in ambient temperature only indicates an alteration in thermogenesis but is not specific for BAT function.

ASSESSMENT OF MAXIMAL THERMOGENIC CAPACITY

A better measure than total daily energy expenditure for assessing BAT function is to consider maximal thermogenic capacity. Maximal thermogenic capacity refers to the greatest quantity of heat that a mouse *can* produce, as opposed to how much heat it produces under free-living conditions. To assess maximal thermogenic capacity a supramaximal dose of a thermogenic drug (usually norepinephrine or the β3-adrenergic receptor agonist CL316243) is administered to an animal to maximally activate BAT. Inevitably, organs other than BAT will also be stimulated in response to adrenergic agonists. In order to identify BAT-specific alterations in thermogenic capacity it is necessary to measure maximal thermogenic capacity at two separate temperatures. First, maximal thermogenic capacity is measured after mice have been acclimated to thermoneutrality to minimize BAT thermogenic capacity. Second, mice are acclimated to 4°C in order to produce a large increase in BAT thermogenic capacity. Ideally the same group of animals will be acclimating sequentially to the two different temperatures, with maximal thermogenic capacity assessed after each acclimation. Alternatively, two separate groups of animals can be used. Cannon and Nedergaard have demonstrated that the difference between norepinephrine stimulated energy expenditure at 4°C and at 30°C is entirely dependent on the presence of UCP1 (Golozoubova et al., 2006), suggesting that this component of thermogenic capacity is BAT dependent. As such, assessment of maximal thermogenic capacity provides a relatively direct measure of BAT function that can be conducted on living animals. Importantly, the actual measurement of thermogenic capacity must be conducted at thermoneutrality in order to turn off the sympathetic nervous tone, regardless of prior acclimation.

Figure 1A shows a typical plot of maximal thermogenic capacity for wild-type mice that have been acclimated to either 5°C or 30°C for 3 weeks prior to measurement. The energy expenditure dependent on BAT is indicated by the vertical arrow between the two norepinephrine stimulated (right hand side) portions of the plots.

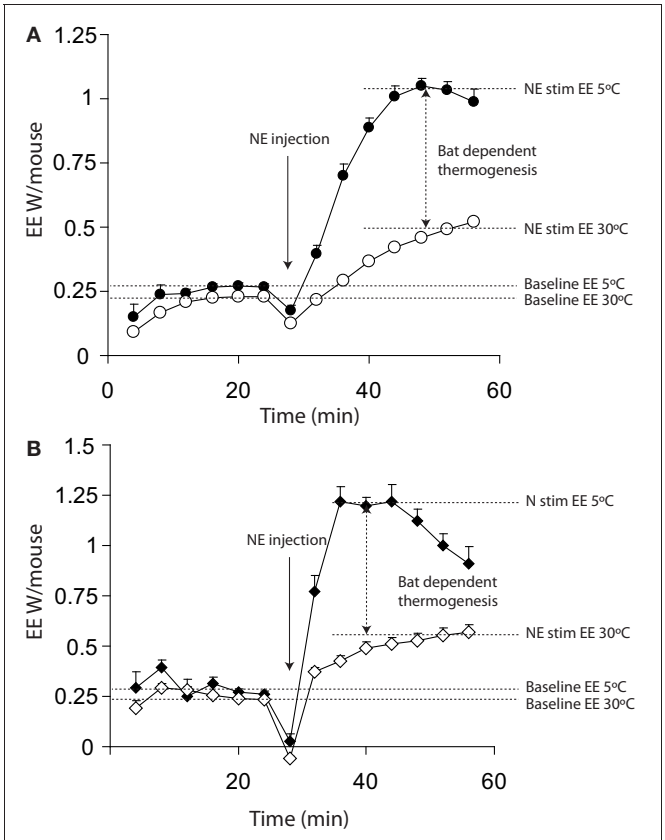


FIGURE 1 | Energy expenditure from 11 warm acclimated (30°C for 3 weeks) and 8 cold acclimated (5°C for 3 weeks) mice. Mice were injected with 1 mg/kg norepinephrine at the time point indicted. All mice were anaesthetized with 60 mg/kg pentobarbital and measured at 30°C, regardless of prior acclimation temperature. **(A)** Energy expenditure uncorrected for lag showing a successive increase in energy expenditure between 0 and 16 (prior to injection but following the mouse being place in the chamber) minutes and 24 and 40 min (post injection with norepinephrine). **(B)** The same data as shown in panel **(A)** corrected for lag, showing apparently more rapid changes in energy expenditure. All mice; male, C57Bl/6, 4 months of age, chamber volume 2.7 l. Energy expenditure measurements conducted using a custom built calorimetry system, using a paramagnetic oxygen analyser and infrared carbon dioxide detector. Flow rate was 0.4 l/min measured on incurrent air to the chambers.

This profile of change in energy expenditure is additionally influenced by two critical factors; the impact of lag and whether the animals are conscious or unconscious (anaesthetized). Failure to consider these factors can significantly affect the results.

THE IMPACT OF LAG

A key factor in any assessment of maximal thermogenic capacity is lag. A lag is a non-specific delay between two events occurring. In the case of calorimetry, lag refers to the time between energy being expended by a mouse and the change in energy expenditure being observed. Importantly, the lag is not a simple linear delay that can be corrected for by realigning data. **Figure 2** demonstrates the energy expenditure that would be observed if a theoretical mouse producing 1 W of energy was placed into chambers of different

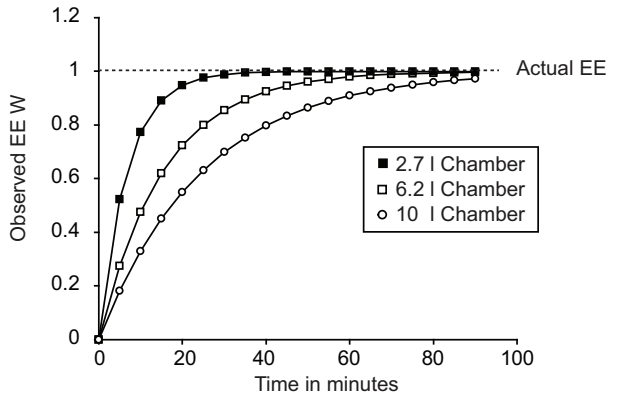


FIGURE 2 | Plot demonstrating the effect of calorimetric lag for different chambers. All cases show the theoretical effect of lag on observed changes in energy expenditure dependent on chamber volume. In all three cases a "mouse" was placed into the chamber at time 0 expending 1 W. Chamber volumes of 2.7 l, 6.2 l, and 10 l correspond to the volumes of a Columbus mouse Oxymax chamber, Columbus CLAMS mouse double-feeder cage and the approximate volume of a Tecniplast mouse cage now used in home-cage calorimetry systems.

sizes with an air flow of 0.4 l/min. The larger the chamber, the longer it takes between a change in energy expenditure occurring and the system coming to equilibrium.

In theory a calorimetry chamber should show a monoexponential relationship between changes in the rate of consumption or production of gases by a mouse and the change in the observed gas concentration within the chamber. The likelihood is that a monoexponential model is an over simplification as calorimeters have multiple separate chambers potentially including the calorimetry chamber itself, tubing between chambers and analysers, drying chambers, and measurement chambers. However, in most systems the calorimetry chamber volume predominates and most of the lag can be accounted for by a simple monoexponential correction.

CORRECTING FOR LAG

Correcting for lag employs an approach called the instantaneous or Z correction (Lighton, 2008). The idea behind the correction is to consider not only the observed energy expenditure at any given time point, but also the rate of change in energy expenditure between the current observation and the previous observation. Mathematically the approach combines the gas concentration observed at a given time point (T_0) and adds to it the derivative of the gas concentrations between the current time point and the previous time point (T_0–T_1), multiplied by a constant. The constant is related to the chamber volume, the flow rate and the time between observations, so a small change in a larger chamber will equate to a large change in smaller chamber. In theory this approach allows energy expenditure values to be generated that represent the actual energy expenditure at any given time point. The following section provides a brief description of how to correct for lag, however, a much more detailed description of the calculations for both energy expenditure and lag correction are provided by Lighton (2008).

Most commercial calorimetry systems do not automatically provide lag corrected energy expenditure. Before explaining how to correct for lag, it is necessary to explain how to derive VO_2 and VCO_2, as these equations are necessary in order to apply lag correction. To calculate the amount of oxygen consumed by the mouse (the VO_2) then the fractional concentration (Fc) of the oxygen entering and leaving the chamber must be known, as must the flow rate (FR) entering and leaving the chamber. Fractional concentrations range between 0 and 1, whereas gas concentrations in most commercial calorimetry systems are usually described as percentages. Percentages can be converted to fractional concentrations by dividing by 100. The equation for VO_2 is shown below in Equation 2:

$$VO_2 = FR_{in}FcO_{2in} - FR_{out}FcO_{2out} \quad (2)$$

In practice, obtaining all the necessary data for calculating VO_2 often poses problems, as while the concentrations of O_2 leaving and entering the chamber will be known (often given as the concentration of O_2 in the room air and concentration of O_2 in the chamber by commercial calorimetry software), only one of the flow rates (either into or out of the chamber) will usually be available. However, it is possible to take advantage of the concentration of nitrogen in the measured gases in order to correct for this. Nitrogen is not produced or consumed by mice, so the flow rate of nitrogen is assumed to be constant. The fractional concentration of nitrogen (FcN_2) can be calculated by subtracting the fractional concentrations of oxygen, carbon dioxide and water from 1. Of note, if systems scrub (remove) water or CO_2, only the fractional concentrations of the non-scrubbed gasses must be subtracted from 1 to give the FcN_2. Given that nitrogen is not produced or consumed, the following equation is valid:

$$FR_{in}FcN_{2in} = FR_{out}FcN_{2out} \quad (3)$$

If Equation 3 is rearranged then the flow rate out of the chamber can be calculated from the fractional concentrations of nitrogen and the flow rate into the chamber:

$$FR_{out} = FR_{in}\frac{FcN_{2in}}{FcN_{2out}} \quad (4)$$

VO_2 can now be calculated when only one flow rate is known (in this case FR_{in}) by substituting Equation 4 into Equation 2 to yield Equation 5.

$$VO_2 = FR_{in}FcO_{2in} - FR_{in}FcO_{2out}\frac{FcN_{2in}}{FcN_{2out}} \quad (5)$$

The VO_2 will be given in the units that the flow rate is expressed in (i.e., ml/min).

The lag correction itself relies on recalculating the FcO_{2out} in Equation 5. Correcting for lag relies on taking the first derivative of the oxygen concentration between two time points (T_0 and T_{-1}) multiplied by a constant and then adding it to the oxygen concentration at T_0. This correction is known as the Z

correction or instantaneous correction. A simplified form of The Z correction is as follows:

$$K\left(FcO_{2outT_0} - FCO_{2inT_{-1}}\right) + FcO_{2outT_0} \quad (6)$$

The corrected Fc_{out} shown in Equation 6 for O_2 can be substituted into the calculation for VO_2 (Equation 5). In theory the value K should be related to the volume of the chamber, the flow rate and the time between measurements according to the Z equation (Lighton, 2008). However, in practice the constant K should be determined empirically by infusing a square wave signal of gas using an infusion pump, as theoretical chamber volumes often differ substantially from observed chamber volumes. The constant should be adjusted manually until the data produces as close an approximation of a square wave as is possible. Application of such a correction would make the observed signals shown in **Figure 2** for the different chamber volumes represent a horizontal line from 0 to 90 min that intercepts the y-axis at 1 W.

Most calorimeters provide gas concentrations in percentages and the following equations are rederivations of the VO_2 and Z corrections above. Equation 7 provides the VO_2 at any given time point and Equation 8 provides the differential VO_2 between a given time point (T_0) and the previous time point (T_{-1}).

$$\text{Flow rate(ml/min)}\frac{\%N_{2out}}{100}\left(\frac{\%O_{2in}}{\%N_2in} - \frac{\%O_{2out}}{\%N_{2out}}\right) \quad (7)$$

$$C\frac{\%N_{2outT_0}}{100}\left(\frac{\%O_{2outT_{-1}}}{\%N_{2outT_{-1}}} - \frac{\%O_{2outT_0}}{\%N_{2outT_0}}\right) \quad (8)$$

Adding Equation 7 (calculated for T_0) and Equation 8 together gives the lag-corrected VO_2 at any given time point in ml/min. In Equation 8, C is a constant that theoretically is the chamber volume divided by the time between T_0 and T_{-1}, with both in the units of the flow rate (e.g., ml for the chamber volume and minutes for time if the flow rate is ml/min). However, as mentioned above, the constant C should be empirically determined. Performing the same calculations for $\%CO_2$ allows lag corrected values for -VCO_2 to be calculated. Once lag-corrected VO_2 and VCO_2 have been determined, the lag-corrected energy expenditure can be calculated using the Weir equation (Equation 1), and the lag corrected RER can be calculated by dividing VCO_2 by VO_2.

IMPACT OF LAG ON MAXIMAL THERMOGENIC CAPACITY DATA

Figure 3A shows the difference between lag corrected and lag uncorrected data from **Figure 1**. As can be seen, the maximal rate of thermogenesis in the uncorrected data is apparently observed at approximately 24 min post injection of norepinephrine. With lag correction, maximal thermogenesis is observed only 12 min post injection. Lag correction is desirable, because chambers of different volumes have different lag periods, thus in different facilities mice may appear to take more or less time to respond to norepinephrine.

Analysis of data from maximal thermogenic capacity is an area that deserves some consideration. In theory the single highest value obtained is the maximal thermogenic capacity; however, it is perhaps prudent to consider the largest three values reached during assessment of maximal thermogenesis in order to have a more stable value. (Lelliott et al., 2006). When analysing maximal thermogenic capacity, lag correction can have a substantial effect. Lag correction of norepinephrine stimulated energy expenditure gives a substantially larger maximal energy expenditure than that observed with uncorrected data (**Figure 3B**) and the difference in energy expenditure (Δ energy expenditure) between basal and stimulated energy expenditure, particularly in the cold, is increased (**Figure 3C**).

THE USE OF CONSCIOUS vs. UNCONSCIOUS ANIMALS FOR MAXIMAL THERMOGENIC CAPACITY MEASUREMENTS

Maximal thermogenic capacity measurements can be conducted in conscious (Meyer et al., 2010) or unconscious (Golozoubova et al., 2006) mice. There are several practical considerations to either approach. First, injecting conscious animals, even with saline, will result in a relatively large increase in energy expenditure due to handling stress (**Figure 4**). Additionally, with conscious mice, physical activity may introduce considerable noise into experiments. These problems are diminished in the stimulated state when utilizing potent thermogenic agents such as norepinephrine, as their effect on energy expenditure is much greater than noise from activity or injection stresses. However, obtaining good baseline values for energy expenditure is likely to be problematic in conscious animals. Minimal observed energy expenditure values (for example the lowest 3 energy expenditure readings observed over a 3 h period) are often used as a measure of basal metabolic rate (BMR) (Meyer et al., 2010). The assumption is that the lowest values will represent periods when mice are inactive and thus BMR can be accurately observed. There are two potential problems with using minimal observed energy expenditure values in free living mice. First, if lag is not corrected for, then the minimal observed energy expenditure values are likely to be overestimated as they will include the effects of energy expenditure that have occurred over a 30+ min period dependent on the cage volume. Secondly, if energy expenditure is corrected for lag, there is a potential to underestimate BMR. Use of the Z correction for lag can result in an over estimation of the change in energy expenditure if there is noise from the oxygen analysers (Lighton, 2008), or the constant K (Equation 6) is too large.

Using unconscious animals removes many of the potential problems seen in conscious mice. Baseline measurements become stable more rapidly as endogenous SNS tone is minimized (**Figure 1A** vs. **Figure 4**). Noise due to processes such as physical activity is also very low. When using unconscious animals, substantial care must be taken regarding the anesthetic of choice. While most anesthetics lead to a reduction in sympathetic tone in mice (hence the requirement to control body temperature during surgery) it is critical that they do not impair sympathetic signaling. Gaseous anesthetics such as isoflurane are known to disrupt adrenergic signaling at the level of receptors (Ohlson et al., 1994). So far, to our knowledge, the only published anesthetic used in maximal thermogenic capacity measurements is pentobarbital. Pentobarbital has a relatively narrow anesthetic window between full sedation and death. It also seems that cold-acclimated mice

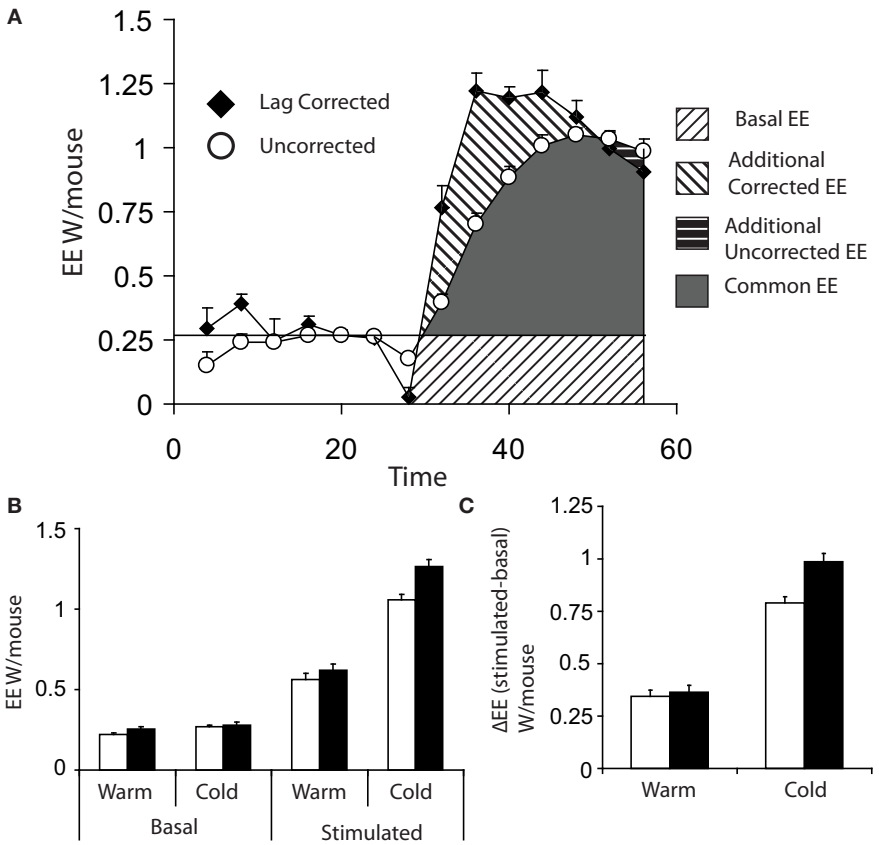


FIGURE 3 | (A) Comparison of energy expenditure plots from **Figures 1A,B** of 8 cold acclimated mice, which have been corrected for lag (black diamonds) or uncorrected (white circles). **(B)** Basal (the average of the last three energy expenditure readings prior to injection) and maximum norepinephrine (NE) stimulated energy expenditure (energy expenditure) (average of the largest three recorded values) based on either lag corrected (black bars) or uncorrected (white bars). **(C)** Δ energy expenditure (maximal stimulated energy expenditure—basal energy expenditure) in response to norepinephrine corrected for lag (black bars) or uncorrected (white bars). All mice; male, C57Bl/6, 4 months of age, chamber volume 2.7 l. Energy expenditure measurements conducted using a custom built calorimetry system, using a paramagnetic oxygen analyser and infrared carbon dioxide detector. Flow rate was 0.4 l/min and measured on incurrent air to the chambers.

are more resistant to the effects of pentobarbital when compared to warm acclimated mice (authors' own observation), making anesthetic dose optimization necessary for any new experimental protocol.

SYMPATHETIC NERVOUS SYSTEM TONE AND THE REGULATION OF BAT

The single most important regulator of BAT function is the SNS (Cannon and Nedergaard, 2004). As such, assessing SNS tone in mice is a technique of considerable value.

CENTRAL REGULATION OF SYMPATHETIC TONE

Considerable work has investigated the pathways that mediate how an organism; (1) detects changes in environmental temperature; (2) relays and integrates information regarding changes in environmental temperature within the central nervous system, and (3) activates thermoregulatory pathways that act to defend core body temperature. A series of excellent reviews have covered central regulation of thermogenesis and fever (Morrison et al., 2008, 2012; Nakamura, 2011).

A series of putative peripheral temperature receptors that belong to the transient receptor potential (TRP) family of cation-selective channels have been identified. TRPA1 (Story et al., 2003) and TRPM8 (Mckemy et al., 2002; Peier et al., 2002) have been suggested to detect noxious and mild cold respectively. Conversely, TRPV1 is suggested to be an extreme heat receptor (Caterina et al., 1997). Finally, TRPV3 (Xu et al., 2002) and TRPV4 (Guler et al., 2002) have been suggested to be able to detect subtle increases in temperature.

Once changes in skin temperature are detected, sensory neurons transmit signals ultimately to the preoptic area of the hypothalamus. The pathway for afferent temperature signals has been shown to be via the dorsal horn (Craig et al., 1994), followed by the lateral parabrachial nucleus (Nakamura and Morrison, 2008). Peripheral temperature signals principally converge on the preoptic area. In addition to signals from peripheral cold receptors, the preoptic area also integrates inflammatory signals, which regulate fever via the production of prostaglandin E2 (Nakamura, 2011) which is sensed by EP3 receptors in the preoptic area (Lazarus et al., 2007). In response to cold, the

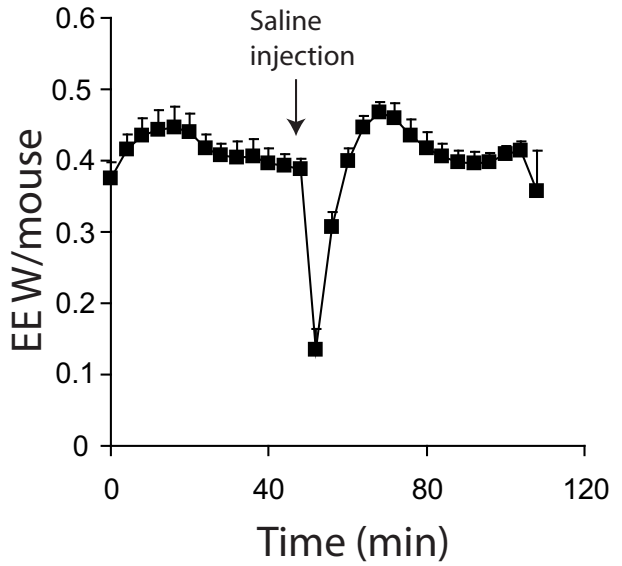


FIGURE 4 | Energy expenditure of 7 mice acclimated and measured at 21°C. All mice were conscious during the experiment. Mice were injected with saline where indicted. All mice; male, C57Bl/6, 4 months of age, chamber volume 6.2 l. Energy expenditure measurements conducted using a custom built calorimetry system, using a paramagnetic oxygen analyser and infrared carbon dioxide detector. Flow rate was 0.4 l/min and measured on incurrent air to the chambers.

preoptic area regulates SNS outflow to peripheral vessels, heart and BAT (Morrison et al., 2012). The preoptic area principally regulates sympathetic tone via inhibition of neurons in the dorsomedial hypothalamus (Dimicco and Zaretsky, 2007). Skin cooling attenuates the inhibitory signal from the preoptic area to the dorsomedial hypothalamus (Nakamura and Morrison, 2007), which is then able to activate premotor neurons in the rostral raphe pallidus which lead to BAT activation via activation of sympathetic preganglionic neurons in the spinal intermediolateral cell column (Morrison et al., 2012).

Ultimately, the critical effector arm of the nervous system for control of BAT is the SNS. Therefore, determining the outflow of the SNS to BAT is of considerable value for identifying the potential mechanistic basis of differences in BAT capacity or function.

MEASUREMENT OF SYMPATHETIC TONE

Three major methods for directly measuring sympathetic tone exist, while there are also several indirect molecular-markers of sympathetic tone that can be used to assess tissue-specific sympathetic activity. In this section we will discuss practical considerations regarding these methods.

DIRECT METHODS FOR ASSESSMENT OF SYMPATHETIC NERVOUS SYSTEM TONE

DIRECT NERVE RECORDING

Direct nerve recording is based on surgically attaching electrodes to nerves subtending BAT (or any other tissue of interest) and recording nerve firing over time (Morrison, 1999; Rahmouni et al., 2005). Once electrodes are attached, recordings can be carried out over period of up to 3 h, during which time stimuli designed to modulate sympathetic nerve signaling can be applied. One such stimulus is to cool the anaesthetized mouse in order to obtain a "cold exposure" response (Morrison, 1999; Whittle et al., 2012). Equally, responses to administration of drugs, either peripherally or directly to specific brain regions via intracranial injection (IC), can be assessed in terms of alterations in SNS tone. By using a combination of the pattern of SNS firing and site-specific IC injections of inhibitors and activators of SNS tone it is also possible to determine which brain regions are responsible for regulating SNS tone to specific tissues and organs (Morrison, 1999). One of the key aspects of such studies is that both sympathetic firing and physiological responses can be simultaneously monitored, with changes in blood pressure heart rate and energy expenditure monitored at the same time as SNS outflow over a time scale of seconds. Direct nerve recordings have been of particular value for investigating the brain regions involved in central control of thermogenesis. For example, as mentioned above, the dorsomedial hypothalamus receives inhibitory inputs from the preoptic area which are alleviated in response to cold. Activation of the dorsomedial hypothalamus promotes activation of BAT via glutamatergic signaling to the rostral raphe pallidus, which in turn activates downstream signals that ultimately converge on BAT. The presence of a tonic γ-aminobutyric acid A Receptor ($GABA_A$) mediated inhibition of neurons in the dorsomedial hypothalamus and its role in controlling BAT SNS outflow was demonstrated by injection of the $GABA_A$ agonist muscimol into the dorsomedial hypothalamus, which prevents SNS outflow to BAT in response to skin cooling (Nakamura and Morrison, 2007). Conversely, injecting the $GABA_A$ receptor antagonist bicuculline into the same nucleus causes increased SNS outflow to BAT (Zaretskaia et al., 2002).

Direct nerve recordings have some very powerful advantages over other methods of assessing SNS tone. Direct nerve recordings have a far higher temporal resolution than other techniques for assessing SNS tone, which has allowed the central pathways regulating BAT function to be mapped out. Additionally, they also provide information about the nature of nerve firing events (frequency and amplitude). As different tissues within organs can possess different SNS activation patterns (Morrison, 2001) direct recordings of single nerve fibers can allow high resolution analysis of SNS activity to a tissue and potentially allow assignment of SNS outflow to specific functions within the tissue.

Direct nerve recordings are limited by several practical considerations. First, recordings can only be performed on anaesthetized animals, limiting the range of physiological stimuli that can be investigated. Second, an expensive electrical recording set up is required. Comprehensive electromagnetic isolation is required in order to allow detection of the extremely low voltages generated by nerves, requiring a dedicated space within an animal facility. Equally a very high degree of technical skill is required to enable appropriate electrode placement. Overall, direct nerve recordings currently remain the provision of dedicated laboratories.

NOREPINEPHRINE TURNOVER USING RADIOACTIVE TRACERS

Norepinephrine turnover relies on the fact that sympathetic nerve endings reuptake norepinephrine, allowing exogenously

administered radioactively-labeled norepinephrine to be accumulated in tissues. The first step in the norepinephrine turnover method for assessing SNS tone is to administer radio-labeled norepinephrine at tracer levels intravenously (IV) to a mouse prior to study. Norepinephrine is then taken up by nerve endings along with endogenous norepinephrine as part of the norepinephrine reuptake system. Following a wash-out period (where excess norepinephrine is lost in urine), groups of animals can be exposed to a given stimuli (e.g., cold) and the rate of loss of labeled norepinephrine from the tissue at different time points can be assessed. Norepinephrine is lost from the tissue when nerves fire, as reuptake of norepinephrine is not 100% efficient. Lost norepinephrine is replaced by newly synthesized and therefore unlabelled norepinephrine. Thus, as sympathetic nerves fire, the tissue-levels of norepinephrine decrease over time and the rate of norepinephrine depletion is assumed to be proportional to the rate of sympathetic nerve firing. This technique assumes that the percentage rate of norepinephrine reuptake is constant across the animals studied, although baseline differences in reuptake can in part be accounted for by the starting levels of labeled norepinephrine within a tissue, as the amount of label initially entering the tissue will be proportional to reuptake. The total amount of norepinephrine in the tissue must be assessed in order to provide mass rates of norepinephrine turnover (i.e., ng/organ/hour) as opposed to purely providing rates of turnover as percentages per hour. Importantly, calculations for the turnover method rely on there being a steady state level of norepinephrine within the tissue. To confirm norepinephrine levels are stable, norepinephrine concentrations in the tissues must be measured at each time point. Measurement of catecholamines has been reviewed extensively elsewhere (Peaston and Weinkove, 2004) and is non-trivial, particularly when using tissue extracts. Isolation of catecholamines is usually performed by acid washed alumina extraction and the concentration is determined either by mass spectrometry (GC-MS) or high performance liquid chromatography (HPLC) coupled to electrochemical detection (Maycock and Frayn, 1987). Although ELISA techniques exist they tend to lack sensitivity compared to GC-MS and HPLC methods.

Measuring sympathetic tone using radioactive tracers has one major advantage over direct sympathetic nerve recordings—it can be performed in conscious animals over periods of time up to at least 24 h. This means that the potential for suppression of endogenous SNS tone by anesthetic is eliminated when compared to the direct nerve recording method. Equally, the effect of physiological challenges such as high-fat feeding can be assessed while the stimulus is actually occurring, as opposed to only studying adaptive responses when using direct nerve recordings.

Conversely, measuring sympathetic tone by norepinephrine turnover has several major disadvantages over direct nerve recordings. Firstly, it is animal intensive; each time point requires a group of animals. Even using a small group (e.g., $n = 4$) for each time point will require at least 12 animals per intervention (e.g., 12 WT and 12 KO) because three time points are necessary. The requirement for three or more time points comes from the fact that norepinephrine turnover should follow a semi-exponential decay profile and 3 time points is the minimum possible to assess if the data fits this profile. Secondly, the capacity to house radioactive, conscious animals within an animal facility is required. The quantity of radioactivity is also substantial—18 to 37 MBQ per kg being a typical level of tracer. Third, the tracer method relies on a steady state level of norepinephrine. For example, acute cold exposure for 24 h will reduce endogenous BAT norepinephrine content by half—invalidating the use of the tracer method (Young et al., 1982). To confirm that norepinephrine levels are at steady state during an experiment, radioactive samples have to be analysed by HPLC or GC-MS requiring an expensive and potentially dedicated piece of equipment.

SYMPATHETIC TONE ASSESSMENT BY TYROSINE HYDROXYLASE INHIBITION

The final method routinely used for measuring SNS activity is the tyrosine hydroxylase inhibition method. Tyrosine hydroxylase inhibitors such as alpha methyl-P-Tyrosine (AMPT) methyl-ester hydrochloride are used to block synthesis of new catecholamines including norepinephrine. Again this technique relies on the fact that norepinephrine reuptake by sympathetic nerve endings is less than 100% efficient. Blocking synthesis of new catecholamines leads to depletion of norepinephrine in the nerve endings within BAT and the rate of disappearance of norepinephrine from the tissue is assumed to be proportional to the rate of sympathetic nerve firing. The fractional turn over rate (based on the slope of the disappearance) is multiplied by the initial norepinephrine level of the baseline group in order to obtain the norepinephrine turn over rate (Young et al., 1982).

The use of AMPT inhibition has almost the same advantages and disadvantages as the tracer technique when compared to the direct nerve recording method. However, unlike the tracer method described above, the inhibitor method has the advantage of being compatible with experiments where there is a non-steady state level of norepinephrine (Young et al., 1982). The major disadvantage of the inhibitor method when compared to the tracer method is that it can only be used for relatively short periods of time (usually up to 6 h dependent on physiological conditions) as over prolonged periods of time tissue norepinephrine stores will be depleted. Furthermore, AMPT reduces tissue catecholamine levels in all tissues, potentially affecting the phenotype under study. Overall, the radioactive tracer method is preferable to the tyrosine hydroxylase inhibition method except in cases where endogenous norepinephrine levels in the tissue under study are not stable.

NON-SPECIFIC METHODS OF ASSESSING SYMPATHETIC ACTIVITY

There are several simple methods that can indicate potential alterations in SNS outflow to BAT. Firstly, measurement of molecular markers of SNS activity in BAT can be used to assess adrenergic signaling. Acute changes in the phosphorylation of proteins such as cAMP response element binding protein (CREB), HSL and p38 mitogen activated protein kinase (p38 MAPK) are all good markers of increased β-adrenergic signaling. An important caveat is that many of these proteins will have their phosphorylation status actively down-regulated after chronic exposure, making the time point studied critical. Measuring the phosphorylation status of

proteins in response to adrenergic stimulation has the advantage that it is practically straightforward, particularly given the commercial availability of good antibodies. However, as mentioned above, assessing phosphorylation status of proteins tends to be applicable only to acute interventions. Longer-term markers of alterations in SNS tone include mRNA markers of thermogenic genes such as UCP1, Elongation of very long fatty acids 3 (Elovl3), Deiodinase 2 (Dio2), Peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α), Bone morphogenetic protein 8b (BMP8b) and lipocalin prostaglandin D synthase (L-PGDS). Importantly, mRNA or protein markers can only provide information about potential changes in the capacity of BAT to generate heat; they do not provide information about how much heat is actually being produced. A second important caveat is that, changes in mRNA expression levels for genes such as UCP1 or PGC1α do not necessarily correlate directly with physiological changes in BAT thermogenic capacity. (Nedergaard and Cannon, 2013). Finally, many genes induced by SNS activation can also be regulated by non-SNS signals such as thiazolidinediones or changes in thyroid hormone levels, making their analyses alone only suggestive of elevated SNS tone.

BETA ADRENERGIC ANTAGONISTS

Beta adrenergic antagonists can be applied to mice to assess if physiological variables such as energy expenditure or molecular markers (see above) of BAT activation are sympathetically mediated. While simple and rapid, beta adrenergic agonists are non-specific and will affect multiple tissues. Furthermore, they will not necessarily distinguish alterations in SNS tone from post-receptor changes in sensitivity to sympathetic tone. Nevertheless, they are simple, cheap and quick and will implicate the SNS at some level in modulating a given phenotype.

ALTERNATIVE SPECIALIZED MEASURES OF BAT ACTIVATION

THERMOGRAPHY AND TEMPERATURE PROBES

BAT produces heat; therefore it is attractive to think that directly measuring heat production in BAT would be a good method for assessing thermogenesis. On a qualitative level this is probably true, as thermogenic agents such as norepinephrine (Hardman and Hull, 1970; Christoffolete et al., 2004), the TRPA1 agonist allyl-isothiocyanate (AITC) (Masamoto et al., 2009), prostaglandin E2 (Nakamura et al., 2002) and bone morphogenetic protein 8b (Whittle et al., 2012) are able to increase BAT temperature as measured by probes implanted into BAT (Hardman and Hull, 1970; Christoffolete et al., 2004; Masamoto et al., 2009) or by thermography of the skin overlaying the interscapular BAT depot (Whittle et al., 2012). Critically, however, neither method is likely to give a good quantitative measure of thermogenesis.

Infusion of norepinephrine has been reported to increase interscapular BAT temperature by 2°C (Hardman and Hull, 1970) or 6°C (Christoffolete et al., 2004). Norepinephrine infusion can increase metabolic rate by up to 300%, dependent on the prior temperature acclimation of the animal. However, not all changes in BAT temperature are linked to changes in metabolic rate. Intragastric administration of AITC causes an increase in BAT temperature of 1°C (Masamoto et al., 2009). However, subsequent measurements of energy expenditure in response to AITC demonstrated no alterations in total energy expenditure (Mori et al., 2011). AITC also causes vasoconstriction (Masamoto et al., 2009), suggesting that the alteration in BAT temperature could be as a result of increased thermal insulation rather than BAT activity. Furthermore, while a 6°C increase in BAT temperature was observed with norepinephrine infusion, this was assessed over only a 60 min period (Christoffolete et al., 2004). It is unlikely that BAT can maintain such a large increase in tissue temperature as this would eventually kill the tissue. Instead, it is probable that alterations in BAT blood flow, which increases more than 4 fold during norepinephrine infusion in rabbits (Hardman and Hull, 1970) and 25 fold in rats (Foster and Frydman, 1978), would ultimately act to reduce BAT temperature. Several practical considerations regarding the use of temperature probes must be considered. Firstly, an increase in BAT temperature should both precede an increase in core body temperature and should also exceed the temperature of the core, in other words there should be a temperature gradient from warmer BAT to a cooler core. An example of a change in BAT temperature preceding a change in core body temperature can be seen in response to chemical stimulation of the dorsomedial hypothalamus (Zaretskaia et al., 2002). If BAT temperature alone is measured, it is important to consider that a change in BAT temperature may occur due to peripheral vasoconstriction or non BAT-mediated increases in core body temperature.

Overall, while an increase in BAT temperature relative to core body temperature indicates that BAT has been activated; it does not provide reliable quantitative information about the degree of activation.

FLUORODEOXYGLUCOSE (^{18}F) POSITRON EMISSION TOMOGRAPHY (FDG-PET)

The use of FDG-PET to assess BAT has come into focus since the rediscovery of BAT in adult humans (Hany et al., 2002; Nedergaard et al., 2007; Cypess et al., 2009; Saito et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). FDG-PET allows the assessment of glucose uptake into tissues *in vivo*. Deoxyglucose is an analogue of glucose that is taken up by glucose transporters but cannot be metabolized. Once taken up, deoxyglucose is phosphorylated and becomes trapped in the tissue, making its levels within a tissue proportionate to glucose transport. FDG-PET has been used in a number of rodent studies to investigate the effects of both pharmacological activators of BAT including CL316243 (Mirbolooki et al., 2011), nicotine and ephedrine (Baba et al., 2007) as well as acute cold exposure for 2–4 h (Baba et al., 2010; Mirbolooki et al., 2011). While FDG-PET does show some dose dependence in response to CL316243 (Mirbolooki et al., 2011), the relationship between FDG-PET uptake and energy expenditure has not been assessed in rodents. The effect of temperature on FDG uptake into BAT has only be assessed for rats in response to acute cold exposure, with 2 h at 8°C causing barely any increase in glucose uptake (Mirbolooki et al., 2011) whereas 4 h at 4°C causes a doubling of glucose uptake (Baba et al., 2010). Importantly, in both the studies of Mirbolooki et al. and Baba et al. BAT thermogenesis would be

expected to occur, highlighting the fact that FDG-PET only allows assessment of glucose uptake which may not mirror thermogenic activity.

While FDG-PET is a potentially valuable technique allowing simultaneous assessment of separate BAT depots within the same organism, FDG-PET has several major limitations. First, due to size limitations FDG-PET studies have focused almost exclusively on rats and larger animals. More importantly, at present no studies have demonstrated a correlation between energy expenditure and FDG uptake into BAT in mice. Furthermore, FDG-PET is very expensive, requiring both access to an appropriate scanner, the ability to synthesise ^{18}F-FDG and the capacity to very rapidly transport ^{18}F-FDG to the site where the animal is under study, given the 109.8 min half life of ^{18}F. Given these limitations, at present, FDG-PET can only be considered as a qualitative and somewhat expensive method to detect BAT activation in rats and larger animals.

GENERAL TECHNIQUES FOR ASSESSING BAT FUNCTION

The techniques for assessing BAT function described so far are not universally available and tend to be restricted to specialized units. However, a number of simple assessments described below can provide helpful indicators of potential alterations in BAT function and thermogenic capacity.

BAT WEIGHT

BAT weight is a complex variable as it can be affected by both long term factors such as alterations in the number of brown adipocytes, but also by acute factors such as nutritional status. As such interpreting BAT weight in the absence of other information (histology, gene expression, and metabolic data) is virtually impossible and its value as anything other than a qualitative variable (i.e., something happened in BAT) is of question. As mentioned below in the histological analysis section, the lipid content of BAT is highly plastic. Acute cold exposure will entirely delipidate BAT, reducing the tissue's weight while its activity is very high (Christoffolete et al., 2004). Conversely, fasting will also reduce BAT weight (**Figure 5**) while its activity is actually diminished. Nevertheless, alterations in BAT weight are simple to assess and should be considered as a marker of potential alterations in BAT, worthy of further investigation.

PAIR FEEDING

Pair feeding is a powerful technique for detecting differences in metabolic rate, particularly where groups of animals have differences in food intake and differences in body weight. Pair feeding is usually conducted by feeding the hyperphagic group the same amount of food as the hypophagic group ate the previous day. If pair feeding does not fully correct a difference in body weight between the groups it is indicative of an alteration in metabolic rate.

When conducting pair feeding it is important to be able to accurately measure food intake. While this sounds simple mice often grind their food, reducing it to fine dust which cannot easily be seen amongst rodent bedding. To accurately assess food intake, replacing bedding with blotting paper (to absorb urine but allow collection of powdered food) and the use of food cups is desirable. Tung et al. describe such a method for analysis of food preference, however, it can also be used for accurate assessment of food intake when only one choice of diet is presented (Tung et al., 2007). However, even a difference in body weight gain between groups of animals that have been pair fed only implies there is altered energy expenditure; whether it is BAT mediated must be confirmed by additional experiments.

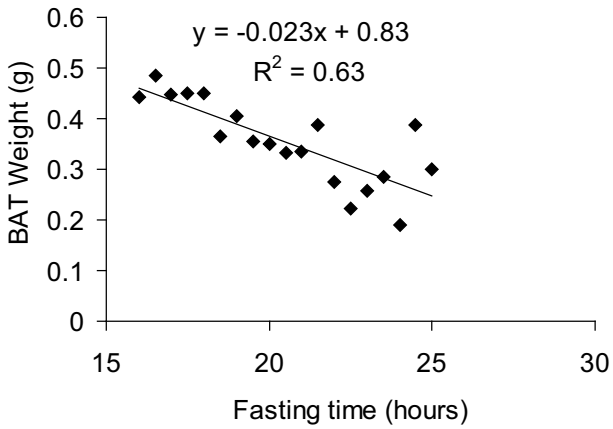


FIGURE 5 | Correlation between BAT weight and fasting time. All mice were fasted overnight for 15 h and then dissected over a period of 8 h, giving a total fasting time ranging from 16 to 24 h for the animals studied. Mice were 7 months old, C57Bl/6 males fed a 60% HFD and acclimated to 5°C for at least 3 weeks prior to culling.

RESPONSE TO FASTING

Weight loss in response to fasting is another potential method for assessing changes in metabolic rate. A greater weight loss in response to an over-night fast is indicative of a hypermetabolic phenotype. In some respects, this is a subset of the pair feeding experiments described above; in this case both groups of animals receive no food instead of the same amount. Again, it is important to note that greater weight loss in response to fasting does not by itself indicate an alteration in BAT function as other organs could be responsible for the observed hypermetabolism.

CHANGES IN CORE BODY TEMPERATURE

The immediate effect of transferring a warm acclimated animal to a cold environment is dependent on (1) the magnitude of the temperature change (2) the species (3) the time of day the transfer occurs [due to the known circadian effects on BAT (Redlin et al., 1992)] and (4) the nutritional status of the animal (Meyer et al., 2010). In general, the effect of transferring mice and rats at any temperature to a substantially colder environment (drop in temperature of 12°C or more) over the first hour is either no reduction in core body temperature or a slight increase of between 0.25 and 1°C (Lomax et al., 1964; Golozoubova et al., 2001; Bratincsak and Palkovits, 2005; Meyer et al., 2010). The slight increase in core body temperature occurring within the first hour is assumed to be related to the combination of increased thermogenesis and peripheral vasoconstriction, which reduces peripheral heat loss.

Over the subsequent 3–4 h of acute cold exposure the response of an animal in terms of core body temperature is heavily dependent on its thermogenic capacity (both shivering and NST). Wild-type mice that have been acclimated to 18°C prior to cold exposure can maintain their core body temperature to within 2°C when exposed to an ambient temperature of 5°C (Golozoubova et al., 2001). When moved to 5°C, wild-type mice that have previously been acclimated to 30°C show a large drop in core body temperature to around 26°C by 3 h (Golozoubova et al., 2001). Mice lacking UCP1 when acclimated to 24°C and then transferred to 5°C rapidly reduce core body temperature, reaching 27°C within 3 h, whereas wild-type mice previously acclimated to 24°C are able to maintain core body temperature at 37°C (Golozoubova et al., 2001). Overall, these results suggest that mice with low thermogenic capacities (either wild-type mice acclimated to 30°C or UCP1 KO mice) do not defend their core body temperature during acute cold challenges as well as mice with greater thermogenic capacities (wild-type mice acclimated to 24°C). However, while a failure to defend core body temperature appropriately can indicate a lack of thermogenic capacity, it is important to note that substantial drops in core body temperature can occur in a regulated manner via the process of torpor. Torpor is an energy preserving state in which mice reduce their core body temperature to as low as 19–20°C and their metabolic rate by 50%. Importantly, mice can spontaneously recover from bouts of torpor lasting several hours (Meyer et al., 2010).

A reduction in core body temperature in response to acute cold exposure is not necessarily a marker of impaired BAT thermogenesis. An inability to undergo shivering thermogenesis can also impact on core body temperature in response to acute cold exposure. The gene sarcolipin has recently been shown to be necessary for appropriate shivering in muscle. Mice lacking the gene sarcolipin, accompanied with surgical removal of interscapular BAT, cannot tolerate cold exposure (Bal et al., 2012) and have a more rapid and sustained loss in core body temperature compared to wild-type mice which have had their interscapular BAT surgically removed.

In general, unless investigation of torpor is a central aim of the study, a core body temperature drop of 10°C is a sensible endpoint for safe termination of an acute cold exposure experiment. From a practical point of view, if an intervention group exhibits a substantially greater loss in core body temperature over a period of 4 h it is sufficient to indicate a potential issue with thermogenesis; however, the involvement in BAT in this process must be determined.

BAT LIPID CONTENT

Acute cold exposure leads to a rapid delipidation of BAT as mobilization and oxidation of endogenous lipid stores outstrips the capacity of brown adipocytes to take up or synthesise lipids (Christoffolete et al., 2004). The first physiological adaptation to cold exposure in BAT is an increase in lipid uptake. On a transcriptional level, expression of the gene lipoprotein lipase (LPL), which is essential for lipid uptake into BAT, is induced as early as 1 h after cold exposure (Mitchell et al., 1992).

Subsequently, *de novo* lipogenesis rates increase, with a significantly increased *de novo* lipogenic capacity detectable at 24 h (Christoffolete et al., 2004). On a gene expression level, at 24 h post-cold exposure there is an increase in glycolytic genes (Yu et al., 2002) and the enzyme acetyl-CoA carboxylase (ACC), which produces the fatty acid synthesis intermediate malonyl-CoA (Yu et al., 2002; Christoffolete et al., 2004). Interestingly, fatty acid synthase (FAS) or SCD1 are not induced as early as ACC, suggesting that levels of ACC in BAT may be the factor limiting *de novo* lipogenic rates.

By 7 days post cold exposure BAT has become largely restocked with lipid, with lipid droplets returning to almost the same size as warm acclimated mice (Christoffolete et al., 2004). By 3 weeks post cold exposure both SCD1 and FAS are induced over control levels. These gene expression changes suggest that BAT increases the rate of lipid restocking of adipose tissue stores and in the longer term allows a maintained high rate of lipid uptake and *de novo* synthesis of lipids.

Brown fat lipid content can be modulated by pharmacological and genetic interventions that impact on BAT in a variety of ways. Treatment with thermogenic compounds such as CL316243 can result in substantial reductions in BAT lipid content within just 2 h (Mirbolooki et al., 2011). Conversely, the UCP1 KO mouse exhibits greater BAT lipid content than wild-type mice at room temperature (21–24°C), assumed to be because of reduced oxidation rates (Enerback et al., 1997).

However, not all changes in BAT lipid content are related to alterations in the metabolic rate of BAT. Lipocalin-Prostaglandin D Synthase KO mice exhibit greater lipid content in BAT under cold exposed (4°C) conditions and express markers of elevated *de novo* lipogenesis in BAT when compared to wild-type controls, despite similar levels of maximal thermogenic capacity (Virtue et al., 2012b). Finally, the elongation of very long chain fatty acids protein 3 (Elovl3) KO mouse exhibits similar lipid content in BAT after acute (3 days) or chronic (3 weeks) exposure to 4°C but has much lower lipid content after housing for 3 weeks at 30°C (Westerberg et al., 2006). The altered lipid level in the BAT of Elovl3 KO mice is complex to interpret. Elovl3 KO mice exhibit altered lipid metabolism and reduced body weight at room temperature (Zadravec et al., 2010) as well as a striking impairment in fur development and skin function (Westerberg et al., 2004). Both these factors could impact on BAT lipid content at 30°C. Firstly, 30°C may not represent thermoneutrality in these mice due to lower insulation. Alternatively, their reduction in peripheral fat mass may affect BAT lipid storage when BAT is inactive at higher temperatures, whereas when BAT must be active (at 4°C) BAT lipid metabolism is spared compared to other organs.

Practically, BAT lipid content can be assessed either by histological morphometry or biochemically. Haematoxylin and eosin staining will stain all non-lipid areas of BAT. Lipid droplets will appear white. Analysing the total white area of a section of BAT by phase analysis will give % lipid content per section. Alternatively, biochemical techniques such as Folch extraction can be used to assess total BAT lipid content (Mclaughlin et al., 2010). The Folch technique relies on accurately weighing BAT and then extracting all the lipid using

solvents (chloroform:methanol) before evaporating the solvents and weighing the lipid.

Overall, there are two major considerations for the analysis of BAT lipid content. First, given the dynamic alterations in BAT lipid accumulation in response to cold exposure, it is important to consider the environmental temperature mice were previously exposed to and for how long they were exposed to it. Second, given that multiple different biological processes can affect lipid levels within BAT, assessment of BAT lipid content cannot be considered indicative of alterations in BAT function when taken in isolation.

GENE EXPRESSION MARKERS

Multiple mRNA markers for activation of BAT and for the browning of WAT exist. The most well-established BAT marker by far is UCP1. It is again important to state that changes in mRNA or even levels of protein markers in BAT will only provide information about the potential thermogenic capacity of BAT, not its activity.

Uncoupling protein 1 (UCP1)

UCP1 is essential for thermogenesis by BAT and its expression is strongly regulated by cold exposure, with UCP1 mRNA expression increasing 10 fold between BAT from animals acclimated to 30°C vs. those acclimated to 4°C for 6 days (Madsen et al., 2010). UCP1 expression is induced in BAT within 3 h of cold exposure (Christoffolete et al., 2004). Acute induction of UCP1 expression seems to be predominantly regulated by adrenergic agonists, with the β3-adrenergic receptor-agonist CL316243 able to increase UCP1 expression 6 fold within 4 h (Cao et al., 2001). Importantly, UCP1 mRNA expression is usually expressed as a concentration per μg of RNA. Assessed in this manner UCP1 mRNA peaks after 4 h in the cold and subsequently diminishes after 1–2 days (Nedergaard and Cannon, 2013). As such changes in UCP1 mRNA alone should be treated with caution. A more physiological measure of BAT thermogenic capacity is total UCP1 protein in a specific BAT depot (Nedergaard and Cannon, 2013).

Elongation of very long chain fatty acids 3 (Elovl3)

Elovl3, also known as Cold Inducible Gene 30 (Cig30) is the most inducible gene with respect to temperature changes in BAT. Cig30 is induced by as much as 200 fold in the BAT of mice after a 3 days exposure to 4°C, following previous acclimation to 30°C (Tvrdik et al., 1997). The function of Elovl3 with respect to BAT function remains unclear due to skin barrier dysfunction in this model (Westerberg et al., 2004).

Peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α) and PGC1β

PGC1β is induced around 3 fold in BAT when comparing mice acclimated to 4°C with mice acclimated to 30°C, whereas PGC1α is induced 5 fold (Lelliott et al., 2006). PGC1α is a key transcriptional co-activator in BAT that regulates mitochondrial biogenesis and brown adipocyte differentiation. PGC1α has been demonstrated to be necessary for the expression of the BAT thermogenic and differentiation program (Lin et al., 2004). PGC1β is also necessary for cold adaptation of BAT (Lelliott et al., 2006). Ablation of PGC1β impairs maximal thermogenic capacity and results in altered mitochondrial morphology (Lelliott et al., 2006). Loss of both PGC1β and PGC1α is lethal, resulting in mitochondria which lack most of their cristae and therefore have greatly diminished capacity for oxidative phosphorylation (Lai et al., 2008).

Deiodinase 2

Deiodinase 2 is an enzyme that converts the thyroid hormone T4 into the active form T3. Thyroid hormone is necessary for full BAT activation and development. Deiodinase 2 is up regulated in states of high BAT activation and is induced by 6 days acclimation to cold exposure by approximately 10 fold (Madsen et al., 2010). Loss of Deiodinase 2 results in impaired capacity for BAT thermogenesis (de Jesus et al., 2001).

SUBCUTANEOUS WHITE ADIPOSE TISSUE AND BRITE CELLS

A large amount of recent interest has focused on brite cells. These are non-canonical brown adipocytes located within predominantly "white" adipose tissue depots. They are developmentally distinct from brown adipocytes found in canonical "brown" depots such as the interscapular depot. Several agents and physiological conditions have been found to promote the development of brite cells including PPARγ activation (Petrovic et al., 2010), exercise (Cao et al., 2011; Bostrom et al., 2012), bone morphogenetic protein 7 (Schulz et al., 2011) and cold exposure. Conversely loss of PPARγ2 greatly reduces brite cell markers in subcutaneous WAT (Virtue et al., 2012c).

While brite cells have been shown *in vitro* to retain much of the functional capacity of a canonical brown adipocyte (Petrovic et al., 2010), a critical and unresolved question is whether they have any impact on whole-organism energy expenditure. As a tissue, even after cold exposure, the inguinal subcutaneous WAT depot expresses only 10% of the UCP1 of the canonical interscapular BAT depot. In mesenteric or epididymal WAT depots the levels of UCP1 are even lower (Nedergaard and Cannon, 2013).

Even extrapolating function from UCP1 mRNA levels, which represents the best-case scenario, it seems improbable that brite cells could contribute to more than a small proportion of total BAT-mediated thermogenesis. However, mRNA or even UCP1 protein levels are only a surrogate for BAT activity. Whether brite cells have sufficient blood supply and innervation to actually produce heat to a meaningful extent *in vivo* remains undetermined. Until such time as the ability of brite cells to actually contribute to whole-body energy expenditure has been demonstrated, considerable caution must be taken before assigning alterations in brite cell number to alterations in energy expenditure.

One important point regarding brite cells is that changes in BAT markers in subcutaneous WAT have a greater dynamic range than those in canonical BAT (Nedergaard and Cannon, 2013). Analysing markers of BAT in subcutaneous WAT may provide a more sensitive readout of changes in sympathetic tone and/or BAT differentiation than analysis of canonical BAT. Therefore, changes in brite cell number/gene expression should not be

ignored as they may be markers of proportionately smaller, but biologically more significant alterations in canonical BAT.

DISCUSSION

This review has attempted to cover practical and theoretical considerations for the study of BAT. Regardless of which field of metabolic research is under investigation, an understanding of the potential for alterations in BAT function to impact on a given phenotype is essential to allow proper interpretation of results in rodent experiments. The capacity for BAT to clear both lipids and glucose from the circulation and to protect animals from high-fat diet induced obesity means that BAT activity affects all aspects of cardiovascular, diabetes, and obesity research. Regardless of whether BAT activation is ever a successful treatment for human metabolic disease, unless BAT and its metabolic consequences are fully understood then it will be almost impossible to translate data from mouse models into humans.

ACKNOWLEDGMENTS

We thank Andrew Whittle, Sergio Rodriguez Cuenca, Chong-Yew Tan and Vivian Pierce for their constructive comments during the writing of this manuscript. We thank the BBSRC, The Sanger Mouse Genetics Project IMPC for funding this work.

REFERENCES

Baba, S., Jacene, H. A., Engles, J. M., Honda, H., and Wahl, R. L. (2010). CT Hounsfield units of brown adipose tissue increase with activation: preclinical and clinical studies. *J. Nucl. Med.* 51, 246–250. doi: 10.2967/jnumed.109.068775

Baba, S., Tatsumi, M., Ishimori, T., Lilien, D. L., Engles, J. M., and Wahl, R. L. (2007). Effect of nicotine and ephedrine on the accumulation of 18F-FDG in brown adipose tissue. *J. Nucl. Med.* 48, 981–986. doi: 10.2967/jnumed.106.039065

Bal, N. C., Maurya, S. K., Sopariwala, D. H., Sahoo, S. K., Gupta, S. C., Shaikh, S. A., et al. (2012). Sarcolipin is a newly identified regulator of muscle-based thermogenesis in mammals. *Nat. Med.* 18, 1857. doi: 10.1038/nm.2897

Bartelt, A., Bruns, O. T., Reimer, R., Hohenberg, H., Ittrich, H., Peldschus, K., et al. (2011). Brown adipose tissue activity controls triglyceride clearance. *Nat. Med.* 17, 200–205. doi: 10.1038/nm.2297

Binczek, E., Jenke, B., Holz, B., Gunter, R. H., Thevis, M., and Stoffel, W. (2007). Obesity resistance of the stearoyl-CoA desaturase-deficient ($scd1^{-/-}$) mouse results from disruption of the epidermal lipid barrier and adaptive thermoregulation. *Biol. Chem.* 388, 405–418. doi: 10.1515/BC.2007.046

Bostrom, P., Wu, J., Jedrychowski, M. P., Korde, A., Ye, L., Lo, J. C., et al. (2012). A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature* 481, 463–468. doi: 10.1038/nature10777

Bratincsak, A., and Palkovits, M. (2005). Evidence that peripheral rather than intracranial thermal signals induce thermoregulation. *Neuroscience* 135, 525–532. doi: 10.1016/j.neuroscience.2005.06.028

Bronnikov, G., Bengtsson, T., Kramarova, L., Golozoubova, V., Cannon, B., and Nedergaard, J. (1999). beta1 to beta3 switch in control of cyclic adenosine monophosphate during brown adipocyte development explains distinct beta-adrenoceptor subtype mediation of proliferation and differentiation. *Endocrinology* 140, 4185–4197. doi: 10.1210/en.140.9.4185

Bronnikov, G., Houstek, J., and Nedergaard, J. (1992). Beta-adrenergic, cAMP-mediated stimulation of proliferation of brown fat cells in primary culture. Mediation via beta 1 but not via beta 3 adrenoceptors. *J. Biol. Chem.* 267, 2006–2013.

Cannon, B., and Nedergaard, J. (2004). Brown adipose tissue: function and physiological significance. *Physiol. Rev.* 84, 277–359. doi: 10.1152/physrev.00015.2003

Cannon, B., and Nedergaard, J. (2009). Thermogenesis challenges the adipostat hypothesis for body-weight control. *Proc. Nutr. Soc.* 68, 401–407. doi: 10.1017/S0029665109990255

Cannon, B., and Nedergaard, J. (2011). Nonshivering thermogenesis and its adequate measurement in metabolic studies. *J. Exp. Biol* 214, 242–253. doi: 10.1242/jeb.050989

Cao, L., Choi, E. Y., Liu, X., Martin, A., Wang, C., Xu, X., et al. (2011). White to brown fat phenotypic switch induced by genetic and environmental activation of a hypothalamic-adipocyte axis. *Cell. Metab.* 14, 324–338. doi: 10.1016/j.cmet.2011.06.020

Cao, W., Medvedev, A. V., Daniel, K. W., and Collins, S. (2001). beta-Adrenergic activation of p38 MAP kinase in adipocytes: cAMP induction of the uncoupling protein 1 (UCP1) gene requires p38 MAP kinase. *J. Biol. Chem.* 276, 27077–27082. doi: 10.1074/jbc.M101049200

Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., and Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389, 816–824. doi: 10.1038/39807

Christoffolete, M. A., Linardi, C. C., De Jesus, L., Ebina, K. N., Carvalho, S. D., Ribeiro, M. O., et al. (2004). Mice with targeted disruption of the Dio2 gene have cold-induced overexpression of the uncoupling protein 1 gene but fail to increase brown adipose tissue lipogenesis and adaptive thermogenesis. *Diabetes* 53, 577–584. doi: 10.2337/diabetes.53.3.577

Craig, A. D., Bushnell, M. C., Zhang, E. T., and Blomqvist, A. (1994). A thalamic nucleus specific for pain and temperature sensation. *Nature* 372, 770–773. doi: 10.1038/372770a0

Cypess, A. M., Lehman, S., Williams, G., Tal, I., Rodman, D., Goldfine, A. B., et al. (2009). Identification and importance of brown adipose tissue in adult humans. *N. Engl. J. Med.* 360, 1509–1517. doi: 10.1056/NEJMoa0810780

de Jesus, L. A., Carvalho, S. D., Ribeiro, M. O., Schneider, M., Kim, S. W., Harney, J. W., et al. (2001). The type 2 iodothyronine deiodinase is essential for adaptive thermogenesis in brown adipose tissue. *J. Clin. Invest.* 108, 1379–1385. doi: 10.1172/JCI13803

Dimicco, J. A., and Zaretsky, D. V. (2007). The dorsomedial hypothalamus: a new player in thermoregulation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 292, R47–R63. doi: 10.1152/ajpregu.00498.2006

Enerback, S., Jacobsson, A., Simpson, E. M., Guerra, C., Yamashita, H., Harper, M. E., et al. (1997). Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. *Nature* 387, 90–94. doi: 10.1038/387090a0

Feldmann, H. M., Golozoubova, V., Cannon, B., and Nedergaard, J. (2009). UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell. Metab.* 9, 203–209. doi: 10.1016/j.cmet.2008.12.014

Foster, D. O., and Frydman, M. L. (1978). Nonshivering thermogenesis in the rat. II. Measurements of blood flow with microspheres point to brown adipose tissue as the dominant site of the calorigenesis induced by noradrenaline. *Can. J. Physiol. Pharmacol* 56, 110–122. doi: 10.1139/y78-015

Golozoubova, V., Cannon, B., and Nedergaard, J. (2006). UCP1 is essential for adaptive adrenergic nonshivering thermogenesis. *Am. J. Physiol. Endocrinol. Metab.* 291, E350–E357. doi: 10.1152/ajpendo.00387.2005

Golozoubova, V., Gullberg, H., Matthias, A., Cannon, B., Vennstrom, B., and Nedergaard, J. (2004). Depressed thermogenesis but competent brown adipose tissue recruitment in mice devoid of all hormone-binding thyroid hormone receptors. *Mol. Endocrinol.* 18, 384–401. doi: 10.1210/me.2003-0267

Golozoubova, V., Hohtola, E., Matthias, A., Jacobsson, A., Cannon, B., and Nedergaard, J. (2001). Only UCP1 can mediate adaptive nonshivering thermogenesis in the cold. *FASEB J.* 15, 2048–2050. doi: 10.1096/fj.00-0536fje

Granneman, J. G., Moore, H. P., Granneman, R. L., Greenberg, A. S., Obin, M. S., and Zhu, Z. (2007). Analysis of lipolytic protein trafficking and interactions in adipocytes. *J. Biol. Chem.* 282, 5726–5735. doi: 10.1074/jbc.M610580200

Granneman, J. G., Moore, H. P., Krishnamoorthy, R., and Rathod, M. (2009). Perilipin controls

lipolysis by regulating the interactions of AB-hydrolase containing 5 (Abhd5) and adipose triglyceride lipase (Atgl). *J. Biol. Chem.* 284, 34538–34544. doi: 10.1074/jbc.M109.068478

Guler, A. D., Lee, H., Iida, T., Shimizu, I., Tominaga, M., and Caterina, M. (2002). Heat-evoked activation of the ion channel, TRPV4. *J. Neurosci.* 22, 6408–6414.

Haemmerle, G., Lass, A., Zimmermann, R., Gorkiewicz, G., Meyer, C., Rozman, J., et al. (2006). Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. *Science* 312, 734–737. doi: 10.1126/science.1123965

Hany, T. F., Gharehpapagh, E., Kamel, E. M., Buck, A., Himms-Hagen, J., and Von Schulthess, G. K. (2002). Brown adipose tissue: a factor to consider in symmetrical tracer uptake in the neck and upper chest region. *Eur. J. Nucl. Med. Mol. Imaging* 29, 1393–1398. doi: 10.1007/s00259-002-0902-6

Hardman, M. J., and Hull, D. (1970). Fat metabolism in brown adipose tissue *in vivo*. *J. Physiol.* 206, 263–273.

Herrington, L. P. (1940). The heat regulation of small laboratory animals at various environmental temperatures. *Am. J. Physiol.* 129, 123–129.

Holm, C. (2003). Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Biochem. Soc. Trans.* 31, 1120–1124. doi: 10.1042/BST0311120

Inokuma, K., Okamatsu-Ogura, Y., Omachi, A., Matsushita, Y., Kimura, K., Yamashita, H., et al. (2006). Indispensable role of mitochondrial UCP1 for antiobesity effect of beta3-adrenergic stimulation. *Am. J. Physiol. Endocrinol. Metab.* 290, E1014–E1021. doi: 10.1152/ajpendo.00105.2005

Kong, W., Stanley, S., Gardiner, J., Abbott, C., Murphy, K., Seth, A., et al. (2003). A role for arcuate cocaine and amphetamine-regulated transcript in hyperphagia, thermogenesis, and cold adaptation. *FASEB J.* 17, 1688–1690. doi: 10.1096/fj.02-0805fje

Lai, L., Leone, T. C., Zechner, C., Schaeffer, P. J., Kelly, S. M., Flanagan, D. P., et al. (2008). Transcriptional coactivators PGC-1alpha and PGC-lbeta control overlapping programs required for perinatal maturation of the heart. *Genes Dev.* 22, 1948–1961. doi: 10.1101/gad.1661708

Lazarus, M., Yoshida, K., Coppari, R., Bass, C. E., Mochizuki, T., Lowell, B. B., et al. (2007). EP3 prostaglandin receptors in the median preoptic nucleus are critical for fever responses. *Nat. Neurosci.* 10, 1131–1133. doi: 10.1038/nn1949

Lelliott, C. J., Medina-Gomez, G., Petrovic, N., Kis, A., Feldmann, H. M., Bjursell, M., et al. (2006). Ablation of PGC-1beta results in defective mitochondrial activity, thermogenesis, hepatic function, and cardiac performance. *PLoS Biol.* 4:e369. doi: 10.1371/journal.pbio.0040369

Lighton, J. R. B. (2008). *Measuring Metabolic Rates: A Manual For Scientists.* Oxford, New York: Oxford University Press. doi: 10.1093/acprof:oso/9780195310610.001.0001

Lin, J., Wu, P. H., Tarr, P. T., Lindenberg, K. S., St-Pierre, J., Zhang, C. Y., et al. (2004). Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha null mice. *Cell* 119, 121–135. doi: 10.1016/j.cell.2004.09.013

Lomax, P., Malveaux, E., and Smith, R. E. (1964). Brain temperatures in the rat during exposure to low environmental temperatures. *Am. J. Physiol.* 207, 736–739.

Madsen, L., Pedersen, L. M., Lillefosse, H. H., Fjaere, E., Bronstad, I., Hao, Q., et al. (2010). UCP1 induction during recruitment of brown adipocytes in white adipose tissue is dependent on cyclooxygenase activity. *PLoS ONE* 5:e11391. doi: 10.1371/journal.pone.0011391

Martínez de Morentin, P. B., Whittle, A. J., Fernø, J., Nogueiras, R., Diéguez, C., Vidal-Puig, A., et al. (2012). Nicotine induces negative energy balance through hypothalamic AMP-activated protein kinase. *Diabetes* 61, 807–817. doi: 10.2337/db11-1079

Masamoto, Y., Kawabata, F., and Fushiki, T. (2009). Intragastric administration of TRPV1, TRPV3, TRPM8, and TRPA1 agonists modulates autonomic thermoregulation in different manners in mice. *Biosci. Biotechnol. Biochem.* 73, 1021–1027. doi: 10.1271/bbb.80796

Maycock, P. F., and Frayn, K. N. (1987). Use of alumina columns to prepare plasma samples for liquid-chromatographic determination of catecholamines. *Clin. Chem.* 33, 286–287.

Mckemy, D. D., Neuhausser, W. M., and Julius, D. (2002). Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* 416, 52–58. doi: 10.1038/nature719

Mclaughlin, B. L., Wells, A. C., Virtue, S., Vidal-Puig, A., Wilkinson, T. D., Watson, C. J., et al. (2010). Electrical and optical spectroscopy for quantitative screening of hepatic steatosis in donor livers. *Phys. Med. Biol.* 55, 6867–6879. doi: 10.1088/0031-9155/55/22/017

Meyer, C. W., Willershauser, M., Jastroch, M., Rourke, B. C., Fromme, T., Oelkrug, R., et al. (2010). Adaptive thermogenesis and thermal conductance in wild-type and UCP1-KO mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 299, R1396–R1406. doi: 10.1152/ajpregu.00021.2009

Mirbolooki, M. R., Constantinescu, C. C., Pan, M. L., and Mukherjee, J. (2011). Quantitative assessment of brown adipose tissue metabolic activity and volume using 18F-FDG PET/CT and β3-adrenergic receptor activation. *EJNMMI Res.* 1, 30. doi: 10.1186/2191-219X-1-30

Mitchell, J. R., Jacobsson, A., Kirchgessner, T. G., Schotz, M. C., Cannon, B., and Nedergaard, J. (1992). Regulation of expression of the lipoprotein lipase gene in brown adipose tissue. *Am. J. Physiol.* 263, E500–E506.

Miyazaki, M., Flowers, M. T., Sampath, H., Chu, K., Otzelberger, C., Liu, X., et al. (2007). Hepatic stearoyl-CoA desaturase-1 deficiency protects mice from carbohydrate-induced adiposity and hepatic steatosis. *Cell. Metab.* 6, 484–496. doi: 10.1016/j.cmet.2007.10.014

Miyoshi, H., Perfield, J. W. 2nd., Souza, S. C., Shen, W. J., Zhang, H. H., Stancheva, Z. S., et al. (2007). Control of adipose triglyceride lipase action by serine 517 of perilipin A globally regulates protein kinase A-stimulated lipolysis in adipocytes. *J. Biol. Chem* 282, 996–1002. doi: 10.1074/jbc.M605770200

Mori, N., Kawabata, F., Matsumura, S., Hosokawa, H., Kobayashi, S., Inoue, K., et al. (2011). Intragastric administration of allyl isothiocyanate increases carbohydrate oxidation via TRPV1 but not TRPA1 in mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 300, R1494–R1505. doi: 10.1152/ajpregu.00645.2009

Morrison, S. F. (1999). RVLM and raphe differentially regulate sympathetic outflows to splanchnic and brown adipose tissue. *Am. J. Physiol.* 276, R962–R973.

Morrison, S. F. (2001). Differential control of sympathetic outflow. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 281, R683–R698.

Morrison, S. F., Madden, C. J., and Tupone, D. (2012). Central control of brown adipose tissue thermogenesis. *Front. Endocrinol.* 3:5. doi: 10.3389/fendo.2012.00005

Morrison, S. F., Nakamura, K., and Madden, C. J. (2008). Central control of thermogenesis in mammals. *Exp. Physiol.* 93, 773–797. doi: 10.1113/expphysiol.2007.041848

Murano, I., Barbatelli, G., Giordano, A., and Cinti, S. (2009). Noradrenergic parenchymal nerve fiber branching after cold acclimatisation correlates with brown adipocyte density in mouse adipose organ. *J. Anat.* 214, 171–178. doi: 10.1111/j.1469-7580.2008.01001.x

Nakamura, K. (2011). Central circuitries for body temperature regulation and fever. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 301, R1207–R1228. doi: 10.1152/ajpregu.00109.2011

Nakamura, K., Matsumura, K., Kaneko, T., Kobayashi, S., Katoh, H., and Negishi, M. (2002). The rostral raphe pallidus nucleus mediates pyrogenic transmission from the preoptic area. *J. Neurosci.* 22, 4600–4610.

Nakamura, K., and Morrison, S. F. (2007). Central efferent pathways mediating skin cooling-evoked sympathetic thermogenesis in brown adipose tissue. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 292, R127–R136. doi: 10.1152/ajpregu.00427.2006

Nakamura, K., and Morrison, S. F. (2008). A thermosensory pathway that controls body temperature. *Nat. Neurosci.* 11, 62–71. doi: 10.1038/nn2027

Nedergaard, J., and Cannon, B. (2013). UCP1 mRNA does not produce heat. *Biochem. Biophys. Acta* 5, 943–949. doi: 10.1016/j.bbalip.2013.01.009

Nedergaard, J., Bengtsson, T., and Cannon, B. (2007). Unexpected evidence for active brown adipose tissue in adult humans. *Am. J. Physiol. Endocrinol. Metab.* 293, E444–E452. doi: 10.1152/ajpendo.00691.2006

Nicholls, D. G. (1974). Hamster brown-adipose-tissue mitochondria. The control of respiration and the proton electrochemical potential gradient by possible physiological effectors of the proton conductance of the inner membrane. *Eur. J. Biochem.* 49, 573–583. doi: 10.1111/j.1432-1033.1974.tb03861.x

Ntambi, J. M., Miyazaki, M., Stoehr, J. P., Lan, H., Kendziorski, C. M., Yandell, B. S., et al. (2002). Loss

of stearoyl-CoA desaturase-1 function protects mice against adiposity. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11482–11486. doi: 10.1073/pnas.132384699

Ohlson, K. B., Mohell, N., Cannon, B., Lindahl, S. G., and Nedergaard, J. (1994). Thermogenesis in brown adipocytes is inhibited by volatile anesthetic agents. A factor contributing to hypothermia in infants? *Anesthesiology* 81, 176–183.

Osuga, J., Ishibashi, S., Oka, T., Yagyu, H., Tozawa, R., Fujimoto, A., et al. (2000). Targeted disruption of hormone-sensitive lipase results in male sterility and adipocyte hypertrophy, but not in obesity. *Proc. Natl. Acad. Sci. U.S.A.* 97, 787–792. doi: 10.1073/pnas.97.2.787

Peaston, R. T., and Weinkove, C. (2004). Measurement of catecholamines and their metabolites. *Ann. Clin. Biochem.* 41, 17–38. doi: 10.1258/000456304322664663

Peier, A. M., Moqrich, A., Hergarden, A. C., Reeve, A. J., Andersson, D. A., Story, G. M., et al. (2002). A TRP channel that senses cold stimuli and menthol. *Cell* 108, 705–715. doi: 10.1016/S0092-8674(02)00652-9

Petrovic, N., Walden, T. B., Shabalina, I. G., Timmons, J. A., Cannon, B., and Nedergaard, J. (2010). Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. *J. Biol. Chem.* 285, 7153–7164. doi: 10.1074/jbc.M109.053942

Rahmouni, K., Morgan, D. A., Morgan, G. M., Mark, A. L., and Haynes, W. G. (2005). Role of selective leptin resistance in diet-induced obesity hypertension. *Diabetes* 54, 2012–2018. doi: 10.2337/diabetes.54.7.2012

Redlin, U., Nuesslein, B., and Schmidt, I. (1992). Circadian changes of brown adipose tissue thermogenesis in juvenile rats. *Am. J. Physiol.* 262, R504–R508.

Rehnmark, S., Nechad, M., Herron, D., Cannon, B., and Nedergaard, J. (1990). Alpha- and beta-adrenergic induction of the expression of the uncoupling protein thermogenin in brown adipocytes differentiated in culture. *J. Biol. Chem.* 265, 16464–16471.

Rothwell, N. J., and Stock, M. J. (1979). A role for brown adipose tissue in diet-induced thermogenesis. *Nature* 281, 31–35. doi: 10.1038/281031a0

Rothwell, N. J., and Stock, M. J. (1983). Luxuskonsumption, diet-induced thermogenesis and brown fat: the case in favour. *Clin. Sci. (Lond.)* 64, 19–23.

Saito, M., Okamatsu-Ogura, Y., Matsushita, M., Watanabe, K., Yoneshiro, T., Nio-Kobayashi, J., et al. (2009). High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes* 58, 1526–1531. doi: 10.2337/db09-0530

Schulz, T. J., Huang, T. L., Tran, T. T., Zhang, H., Townsend, K. L., Shadrach, J. L., et al. (2011). Identification of inducible brown adipocyte progenitors residing in skeletal muscle and white fat. *Proc. Natl. Acad. Sci. U.S.A.* 108, 143–148. doi: 10.1073/pnas.1010929108

Seale, P., Bjork, B., Yang, W., Kajimura, S., Chin, S., Kuang, S., et al. (2008). PRDM16 controls a brown fat/skeletal muscle switch. *Nature* 454, 961–967. doi: 10.1038/nature07182

Sell, H., Berger, J. P., Samson, P., Castriota, G., Lalonde, J., Deshaies, Y., et al. (2004). Peroxisome proliferator-activated receptor gamma agonism increases the capacity for sympathetically mediated thermogenesis in lean and ob/ob mice. *Endocrinology* 145, 3925–3934. doi: 10.1210/en.2004-0321

Shechtman, O., Papanek, P. E., and Fregly, M. J. (1990). Reversibility of cold-induced hypertension after removal of rats from cold. *Can. J. Physiol. Pharmacol.* 68, 830–835. doi: 10.1139/y90-126

Silva, J. E. (2006). Thermogenic mechanisms and their hormonal regulation. *Physiol. Rev.* 86, 435–464. doi: 10.1152/physrev.00009.2005

Skarulis, M. C., Celi, F. S., Mueller, E., Zemskova, M., Malek, R., Hugendubler, L., et al. (2010). Thyroid hormone induced brown adipose tissue and amelioration of diabetes in a patient with extreme insulin resistance. *J. Clin. Endocrinol. Metab.* 95, 256–262. doi: 10.1210/jc.2009-0543

Souza, S. C., Christoffolete, M. A., Ribeiro, M. O., Miyoshi, H., Strissel, K. J., Stancheva, Z. S., et al. (2007). Perilipin regulates the thermogenic actions of norepinephrine in brown adipose tissue. *J. Lipid. Res.* 48, 1273–1279. doi: 10.1194/jlr.M700047-JLR200

Steiner, G., and Evans, S. (1976). Effect of serotonin on brown adipose tissue and on its sympathetic neurons. *Am. J. Physiol.* 231, 34–39.

Story, G. M., Peier, A. M., Reeve, A. J., Eid, S. R., Mosbacher, J., Hricik, T. R., et al. (2003). ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* 112, 819–829. doi: 10.1016/S0092-8674(03)00158-2

Swoap, S. J., Li, C., Wess, J., Parsons, A. D., Williams, T. D., and Overton, J. M. (2008). Vagal tone dominates autonomic control of mouse heart rate at thermoneutrality. *Am. J. Physiol. Heart Circ. Physiol.* 294, H1581–H1588. doi: 10.1152/ajpheart.01000.2007

Takano, T., Honma, T., Motohashi, Y., and Kobayashi, Y. (1987). Streptozotocin diabetes in rats after acclimation to cold environment. *Prev. Med.* 16, 63–69. doi: 10.1016/0091-7435(87)90006-5

Timmons, J. A., Wennmalm, K., Larsson, O., Walden, T. B., Lassmann, T., Petrovic, N., et al. (2007). Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. *Proc. Natl. Acad. Sci. U.S.A.* 104, 4401–4406. doi: 10.1073/pnas.0610615104

Tung, Y. C., Rimmington, D., O'Rrahilly, S., and Coll, A. P. (2007). Pro-opiomelanocortin modulates the thermogenic and physical activity responses to high-fat feeding and markedly influences dietary fat preference. *Endocrinology* 148, 5331–5338. doi: 10.1210/en.2007-0797

Tvrdik, P., Asadi, A., Kozak, L. P., Nedergaard, J., Cannon, B., and Jacobsson, A. (1997). Cig30, a mouse member of a novel membrane protein gene family, is involved in the recruitment of brown adipose tissue. *J. Biol. Chem.* 272, 31738–31746. doi: 10.1074/jbc.272.50.31738

Ukropec, J., Anunciado, R. P., Ravussin, Y., Hulver, M. W., and Kozak, L. P. (2006). UCP1-independent thermogenesis in white adipose tissue of cold-acclimated Ucp1-/- mice. *J. Biol. Chem.* 281, 31894–31908. doi: 10.1074/jbc.M606114200

van Marken Lichtenbelt, W. D., Vanhommerig, J. W., Smulders, N. M., Drossaerts, J. M., Kemerink, G. J., Bouvy, N. D., et al. (2009). Cold-activated brown adipose tissue in healthy men. *N. Engl. J. Med.* 360, 1500–1508. doi: 10.1056/NEJMoa0808718

Virtanen, K. A., Lidell, M. E., Orava, J., Heglind, M., Westergren, R., Niemi, T., et al. (2009). Functional brown adipose tissue in healthy adults. *N. Engl. J. Med.* 360, 1518–1525. doi: 10.1056/NEJMoa0808949

Virtue, S., Even, P., and Vidal-Puig, A. (2012a). Below thermoneutrality, changes in activity do not drive changes in total daily energy expenditure between groups of mice. *Cell. Metab.* 16, 665–671. doi: 10.1016/j.cmet.2012.10.008

Virtue, S., Feldmann, H., Christian, M., Tan, C. Y., Masoodi, M., Dale, M., et al. (2012b). A new role for lipocalin prostaglandin d synthase in the regulation of brown adipose tissue substrate utilization. *Diabetes* 61, 3139–3147. doi: 10.2337/db12-0015

Virtue, S., Masoodi, M., Velagapudi, V., Tan, C. Y., Dale, M., Suorti, T., et al. (2012c). Lipocalin prostaglandin D synthase and PPARgamma2 coordinate to regulate carbohydrate and lipid metabolism *in vivo*. *PLoS ONE* 7:e39512. doi: 10.1371/journal.pone.0039512

Westerberg, R., Mansson, J. E., Golozoubova, V., Shabalina, I. G., Backlund, E. C., Tvrdik, P., et al. (2006). ELOVL3 is an important component for early onset of lipid recruitment in brown adipose tissue. *J. Biol. Chem.* 281, 4958–4968. doi: 10.1074/jbc.M511588200

Westerberg, R., Tvrdik, P., Unden, A. B., Mansson, J. E., Norlen, L., Jakobsson, A., et al. (2004). Role for ELOVL3 and fatty acid chain length in development of hair and skin function. *J. Biol. Chem.* 279, 5621–5629. doi: 10.1074/jbc.M310529200

Whittle, A. J., Carobbio, S., Martins, L., Slawik, M., Hondares, E., Vazquez, M. J., et al. (2012). BMP8B increases brown adipose tissue thermogenesis through both central and peripheral actions. *Cell* 149, 871–885. doi: 10.1016/j.cell.2012.02.066

Xu, H., Ramsey, I. S., Kotecha, S. A., Moran, M. M., Chong, J. A., Lawson, D., et al. (2002). TRPV3 is a calcium-permeable temperature-sensitive cation channel. *Nature* 418, 181–186. doi: 10.1038/nature00882

Xue, Y., Petrovic, N., Cao, R., Larsson, O., Lim, S., Chen, S., et al. (2009). Hypoxia-independent angiogenesis in adipose tissues during cold acclimation. *Cell. Metab.* 9, 99–109. doi: 10.1016/j.cmet.2008.11.009

Young, J. B., Saville, E., Rothwell, N. J., Stock, M. J., and Landsberg, L. (1982). Effect of diet and cold exposure on norepinephrine turnover in brown adipose tissue of the rat.

J. Clin. Invest. 69, 1061–1071. doi: 10.1172/JCI110541

Yu, X. X., Lewin, D. A., Forrest, W., and Adams, S. H. (2002). Cold elicits the simultaneous induction of fatty acid synthesis and beta-oxidation in murine brown adipose tissue: prediction from differential gene expression and confirmation *in vivo*. *FASEB J.* 16, 155–168. doi: 10.1096/fj.01-0568com

Zadravec, D., Brolinson, A., Fisher, R. M., Carneheim, C., Csikasz, R. I., Bertrand-Michel, J., et al. (2010). Ablation of the very-long-chain fatty acid elongase ELOVL3 in mice leads to constrained lipid storage and resistance to diet-induced obesity. *FASEB J.* 24, 4366–4377. doi: 10.1096/fj.09-152298

Zaretskaia, M. V., Zaretsky, D. V., Shekhar, A., and Dimicco, J. A. (2002). Chemical stimulation of the dorsomedial hypothalamus evokes non-shivering thermogenesis in anesthetized rats. *Brain Res* 928, 113–125. doi: 10.1016/S0006-8993(01)03369-8

Zimmermann, R., Strauss, J. G., Haemmerle, G., Schoiswohl, G., Birner-Gruenberger, R., Riederer, M., Lass, A., Neuberger, G., Eisenhaber, F., Hermetter, A., et al. (2004). Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science* 306, 1383–1386. doi: 10.1126/science.1100747

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 06 February 2013; accepted: 16 May 2013; published online: 04 June 2013.

*Citation: Virtue S and Vidal-Puig A (2013) Assessment of brown adipose tissue function. Front. Physiol. **4**:128. doi: 10.3389/fphys.2013.00128*

This article was submitted to Frontiers in Integrative Physiology, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 08 April 2013
doi: 10.3389/fphys.2013.00064

Detection of thermogenesis in rodents in response to anti-obesity drugs and genetic modification

Jonathan R. S. Arch* and Paul Trayhurn

Clore Laboratory, University of Buckingham, Buckingham, UK

Edited by:
Patrick C. Even, AgroParisTech, France

Reviewed by:
Gema Frühbeck, University of Navarra, Spain
Paul MacLean, University of Colorado Anschutz Medical Campus, USA

****Correspondence:***
Jonathan R. S. Arch, Clore Laboratory, University of Buckingham, Hunter Street, Buckingham MK18 1EG, UK.
e-mail: jon.arch@buckingham.ac.uk

Many compounds and genetic manipulations are claimed to confer resistance to obesity in rodents by raising energy expenditure. Examples taken from recent and older literature, demonstrate that such claims are often based on measurements of energy expenditure after body composition has changed, and depend on comparisons of energy expenditure divided by body weight. This is misleading because white adipose tissue has less influence than lean tissue on energy expenditure. Application of this approach to human data would suggest that human obesity is usually due to a low metabolic rate, which is not an accepted view. Increased energy expenditure per animal is a surer way of demonstrating thermogenesis, but even then it is important to know whether this is due to altered body composition (repartitioning), or increased locomotor activity rather than thermogenesis *per se*. Regression analysis offers other approaches. The thermogenic response to some compounds has a rapid onset and so cannot be due to altered body composition. These compounds usually mimic or activate the sympathetic nervous system. Thermogenesis occurs in, but may not be confined to, brown adipose tissue. It should not be assumed that weight loss in response to these treatments is due to thermogenesis unless there is a sustained increase in 24-h energy expenditure. Thyroid hormones and fibroblast growth factor 21 also raise energy expenditure before they affect body composition. Some treatments and genetic modifications alter the diurnal rhythm of energy expenditure. It is important to establish whether this is due to altered locomotor activity or efficiency of locomotion. There are no good examples of compounds that do not affect short-term energy expenditure but have a delayed effect. How and under what conditions a genetic modification or compound increases energy expenditure influences the decision on whether to seek drugs for the target or take a candidate drug into clinical studies.

Keywords: thermogenesis, energy expenditure, leanness, anti-obesity drug, genetically modified mouse, sympathomimetic, brown adipose tissue, leptin

INTRODUCTION

Despite the continuing rise in the worldwide prevalence of obesity and the vast sales that a safe and effective drug for this disorder might achieve, many pharmaceutical companies have been disinclined to invest in research and development in this field in recent years because the task seemed near impossible. Following the withdrawal of fenfluramine and dexfenfluramine in 1997, primarily because they caused heart valve disease, sibutramine, and rimonabant offered a glimmer of hope, but either they never reached the US or European markets, or they were soon withdrawn owing to adverse cardiovascular or CNS side-effects. Only the pancreatic lipase inhibitor orlistat remains for long term pharmacotherapy, and its efficacy (about 3 kg weight loss compared to placebo when used at its highest approved dose) is limited (Rucker et al., 2007).

Some optimism has returned recently following the approval by the US Food and Drug Administration (FDA) of the combination of the anti-epileptic drug topiramate in combination with the "anorectic" drug phentermine under the brand name Qsymia. The European Medicines Agency has rejected Qsymia, however. Soon after its approval of Qsymia, the FDA also approved the selective $5HT_{2C}$ receptor agonist lorcaserin (Wong et al., 2012). Looking to the future, approval of the glucagon-like peptide-1 analogue liraglutide may be helped by it being already marketed for the treatment of type 2 diabetes, albeit at a lower dose than that being evaluated in Phase III clinical trials for obesity.

Apart from orlistat, the primary mechanism of which is to reduce energy absorption rather than intake, these drugs have all been perceived as anorectic agents. Is this entirely true? Studies in rodents suggest that the anti-obesity effects of many "anorectic" drugs are partly, or even entirely, due to increased energy expenditure ("thermogenesis") (Arch, 1981; Day and Bailey, 1998; Picard et al., 2000; Herling et al., 2008). There is nothing new in the concept of thermogenic drugs: two of the earliest drugs for obesity of the scientific era—dinitrophenol and thyroid hormones—stimulate thermogenesis (Clapham and Arch, 2007).

It is important to understand how evidence that compounds are thermogenic in rodents has been obtained and whether this evidence translates to humans, because the question "Is it anorectic or thermogenic?" will continue to be asked of new drugs, drug candidates and drug targets. This question is especially pertinent

because interest in thermogenic drugs and drug targets has been rekindled by new evidence that brown adipose tissue can be active in adult humans and the discovery of new targets for drugs that might augment and activate brown adipose tissue (Fruhbeck et al., 2009; Wu et al., 2011; Bostrom et al., 2012; Fournier et al., 2012; Ye et al., 2012). The role of brown adipose tissue is to oxidise fat without coupling the energy released to the synthesis of ATP. The purpose of this uncoupling is either to produce heat (in which case fat loss can be seen as a by-product) or to regulate body fat stores (with thermogenesis as a by-product). Thus, drugs that target brown adipose tissue should be thermogenic.

The aim of this article is to discuss how thermogenesis in response to treatment with a drug can be detected. In addition, since targets for thermogenic drugs are often validated by investigating the phenotype of genetically modified mice, it considers how to ascertain whether leanness in a genetically modified animal is associated with increased energy expenditure. Examples of various approaches are taken from both old and recent literature. We argue that some of the claims are unjustified. The issue of how to compare the energy expenditure of lean and obese rodents has been discussed extensively elsewhere (Arch et al., 2006; Butler and Kozak, 2010; Kaiyala et al., 2010; Cannon and Nedergaard, 2011; Even and Nadkarni, 2012; Tschop et al., 2012; Speakman, 2013), so we shall not repeat all the arguments, but raise points that others may not have addressed. We are among those who object strongly to the expression of energy expenditure relative to body weight or body weight$^{0.75}$, for the purpose of comparing lean and obese rodents. This is standard practice in most journals and apparently accepted without question by most referees, but it is often misleading or simply wrong—particularly and paradoxically since intake is almost invariably expressed on a "per animal" basis. Thus, a continuing theme is the dismissal of claims based on this practice. The claims that we have selected to dismiss are merely examples, often taken from recent literature. A number of other examples have been described in previous publications (Butler and Kozak, 2010; Arch, 2011), but even taking the articles together, these are only the tip of the iceberg. The translation of studies in rodents to humans is also briefly considered.

ACUTE STIMULATION OF ENERGY EXPENDITURE

RAPID ONSET RESPONSE TO COMPOUNDS

Sympathomimetic agents

It is easiest to make the case for a compound being thermogenic if energy expenditure rises rapidly, ideally within minutes after its administration. This has two advantages over other types of evidence: energy expenditure prior to administration of the compound provides a baseline control, and interpretation of data is not complicated by changes in body weight or composition, because these are no different for the compound- and vehicle-treated animals.

Classic examples of such evidence are the rises in energy expenditure within an hour (or minutes if given intravenously) following administration of noradrenaline, and sympathomimetic compounds such as phentermine, ephedrine and β_3-adrenoceptor agonists (Arch, 1981; Arch et al., 1982, 1984; Wilson et al., 1984; Holloway et al., 1991; Granneman et al., 2003; Kong et al., 2004). The rapid increases in energy expenditure and brown adipose tissue temperature elicited by caffeine (Arch et al., 1987; Yoshioka et al., 1990), theophylline (Strubelt and Siegers, 1969), green tea (Dulloo et al., 2000; Choo, 2003) and nicotine (Wellman et al., 1986; Collins et al., 1996a) are also probably mainly a consequence of their raising sympathetic activity, though other mechanisms—including increased locomotor activity in response to caffeine—may contribute (Arch et al., 1987). Sibutramine similarly elicits a slightly delayed, increase in energy expenditure in rodents as a consequence of it activating the sympathetic nervous system. It took about an hour for this effect to become statistically significant following intraperitoneal administration to rats, presumably because sibutramine must increase synaptic serotonin and noradrenaline concentrations in the hypothalamus before the sympathetic nervous system is activated. The simultaneous injection of the noradrenaline re-uptake inhibitor nisoxetine and the serotonin re-uptake inhibitor fluoxetine stimulated energy expenditure with a similar delay (Connoley et al., 1999). A more recent example is that intracerebroventricular administration of bone morphogenetic protein 8B elicited an increase in sympathetic activity in brown adipose tissue and in core temperature within an hour, but energy expenditure was not measured directly (Whittle et al., 2012). By contrast, zinc-α_2-glycoprotein (ZAG), despite being claimed to be a β_3-adrenoceptor agonist (Russell et al., 2002; Russell and Tisdale, 2012a), did not elicit a rapid rise in energy expenditure in our hands (Wargent et al., 2013), and has not been reported to do so by others. Nevertheless, it may raise energy expenditure over a period of days and reduce body weight and fat independently of any effect on food intake (Russell and Tisdale, 2011a,b).

A possible reason why sympathomimetic agents have such a marked thermogenic effect is that they stimulate the mobilization of fatty acids as well as their combustion. Thus, a β_3-adrenoceptor agonist stimulated thermogenesis in mice that expressed the β_3-adrenoceptor in brown and white adipose tissue (and no other tissue), but it had little effect when the β_3-adrenoceptor was expressed in brown adipose tissue only—the brown adipocytes seemed to be starved of fuel to burn (Grujic et al., 1997). Similarly, antilipolytic agents, such as nicotinic acid, reduce thermogenesis in response to catecholamines and β-adrenoceptor agonists (Kennedy and Ellis, 1969; Lafrance et al., 1979; Schiffelers et al., 1998), and the thermogenic effects of β_3-adrenoceptor agonists are less prolonged in lean or lipoatrophic mice than in obese mice (Arch and Ainsworth, 1983a; Gavrilova et al., 2000).

It is reasonable to consider whether any compound that elicits a rapid increase in energy expenditure does so by activating the sympathetic nervous system. One such compound is the cannabinoid 1-receptor (CB1-R) antagonist rimonabant, which elicits a rapid increase in energy expenditure in rats (Herling et al., 2008; Kunz et al., 2008), though this has not been demonstrated in humans. Antagonism of the CB1-R promotes noradrenaline release at peripheral sympathetic nerves (Marsicano and Lutz, 2006; Mnich et al., 2010) and activates sympathetic activity via a central mechanism (Verty et al., 2009). It is therefore possible that the acute thermogenic effect of rimonabant in rodents is mediated by the sympathetic nervous system. Other examples of rapid

thermogenic responses that are probably mediated by raised sympathetic activity are those to a catalytic antibody that hydrolyzed the octanoyl moiety of ghrelin (Mayorov et al., 2008; Arch, 2011), to amylin (Osaka et al., 2008) and to thyrotrophin-releasing hormone (Schuhler et al., 2007). In the case of thyrotrophin-releasing hormone, evidence for the involvement of the sympathetic nervous system is that its intracerebroventricular infusion increased noradrenaline turnover in brown adipose tissue, and thermogenesis was suppressed by sympathetic denervation of brown adipose tissue (Shintani et al., 2005).

The thermogenic effects of β_3-adrenoceptor agonists and amylin, and the anti-obesity effect of ZAG are reduced or prevented by the β-adrenoceptor antagonist propranolol (Arch and Ainsworth, 1983a; Arch et al., 1991; Osaka et al., 2008; Russell and Tisdale, 2012a,b). We have suggested that the anti-obesity effect of ZAG may be due to central activation of the sympathetic nervous system, rather than direct activation of the β_3-adrenoceptor and this is why its anti-obesity effect is blocked by propranolol (Wargent et al., 2013). Propranolol also blocked thermogenesis in response to the centrally acting sympathomimetic agents caffeine and theophylline (Strubelt and Siegers, 1969). Doses of β-adrenoceptor antagonists have to be high to block the rodent β_3-adrenoceptor and there is therefore a risk that they might elicit non-β-adrenoceptor-mediated effects.

Alternative approaches to investigating the role of the sympathetic nervous system can be illustrated by considering studies on leptin. Intracerebroventricular administration of leptin increases energy expenditure in *ob/ob* mice within 3 h (Mistry et al., 1997). In rats, intracerebroventricular injection of leptin increased body temperature after an hour; peripheral administration took 3 h (Luheshi et al., 1999). Subcutaneous administration has been shown to increase energy expenditure within 12 h in suckling rats (Stehling et al., 1996) and 2 days when infused by minipump in normal mice (Asensio et al., 2008). Leptin increases noradrenaline turnover in brown and white adipose tissue (Collins et al., 1996b) and electrical activity in sympathetic nerves (Dunbar et al., 1997; Hausberg et al., 2002). None of this shows whether increased sympathetic activity is responsible for any of the thermogenic activity of leptin, however. Better evidence is that the thermogenic effect on days 2–6 of subcutaneously infused leptin was reduced by about 50% in mice that lack all three β-adrenoceptors (betaless mice) (Asensio et al., 2008). Work in Lep^{ob}/Lep^{ob} mice that also lack UCP-1 suggests that elevation of plasma tri-iodothyronine by leptin and activation of sarcoendoplasmic reticulum Ca^{2+} ATPase may contribute to some of the remaining thermogenic effect of leptin (Ukropec et al., 2006b). Studies on the role of the sympathetic nervous system in the anti-obesity effect of leptin are described in section Detection of Non-Acute Thermogenesis.

Despite these inconclusive results with leptin, it is advisable to investigate whether a prospective thermogenic anti-obesity compound is as effective in sympathectomised, betaless, or individual β-adrenoceptor knockout mice as it is in normal mice. This information may help in the design of clinical studies (see section Translation of Rodent Findings to Humans). It might also be useful to evaluate compounds in mice that lack brown adipose tissue (Lowell et al., 1993), though it should not be assumed that all sympathetically-mediated thermogenesis in rodents is in brown adipose tissue (Thurlby and Ellis, 1986). Even those who argue that all *adaptive* thermogenesis is in brown adipose tissue find that much of the thermogenic response to noradrenaline in mice that are fed on a normal diet and housed at thermoneutrality remains in mice that lack uncoupling protein-1 (UCP-1), thereby lacking the defining protein of brown adipose tissue (Feldmann et al., 2009). They argue that the UCP-1-independent thermogenic effect of noradrenaline is a pharmacological, non-adaptive effect that takes place in tissues other than brown adipose tissue, rather than a physiological effect (Cannon and Nedergaard, 2011). These arguments are consistent with adaptive thermogenesis being exclusive to brown adipose tissue (Cannon and Nedergaard, 2011) and with reports that thermogenic doses of the β-adrenoceptor agonist isoprenaline and the sympathomimetic drug ephedrine failed to activate brown adipose tissue in humans, although cold exposure did (Cypess et al., 2012; Vosselman et al., 2012). A non-physiological mechanism of action is less attractive in a drug than a physiological mechanism. On the other hand, it seems unlikely that thermogenesis in tissues other than brown adipose tissue is entirely non-physiological, because exposure to cold for 4 days increased triacylglycerol/fatty acid substrate cycling in white as well as brown adipose tissue (Brooks et al., 1983). Other substrate cycles—sometimes called "futile" cycles—may also be stimulated in the cold, even though their primary function may not be thermogenesis. UCP-1 knockout mice are able to increase their response to cold exposure when acclimated to cold, and there is evidence that this is partly due to increased oxidative capacity and ATP utilization in white adipose tissue (Meyer et al., 2010; Ukropec et al., 2006a). Cold exposure (for 2.5 days) also increased fructose-6-phosphate/fructose-1,6-bisphosphate cycling in skeletal muscle but this might have been a consequence of shivering *in vivo*, even though cycling was measured *in vitro* (Challis et al., 1985). On the other hand, when A/J mice were fed on a high fat diet, soleus muscles taken from them had an increased oxygen consumption, which cannot be attributed to shivering (Kus et al., 2008). Moreover, mice in which sarcolipin is absent from skeletal muscle were less able than wild-type mice to defend their body temperature in the cold, even when the mice are treated with curare to prevent shivering, suggesting that futile pumping of Ca^{2+} may play a role in thermogenesis (Bal et al., 2012). Finally, it is worth noting that by contrast with the recent reports that isoprenaline and ephedrine stimulated thermogenesis without activating brown fat in humans (Cypess et al., 2012; Vosselman et al., 2012), noradrenaline stimulated thermogenesis in both brown adipose tissue and the hind limb (primarily skeletal muscle) in rodents (Thurlby and Ellis, 1986), suggesting that brown fat as well as other tissues is physiologically relevant in rodents.

Non-sympathomimetic mechanisms

Non-sympathetic mechanisms may also elicit rapid rises in energy expenditure. Sympathomimetic compounds stimulate thermogenesis primarily via β-adrenoceptors and $G\alpha_s$. One would therefore expect that activation of any $G\alpha_s$-coupled receptor in brown adipose tissue should rapidly activate energy expenditure.

The bile acid receptor TGR5 is an example of such a receptor. Agonists of TGR5 increase the concentration of cyclic AMP in isolated brown adipocytes within 1 h (Watanabe et al., 2006). Surprisingly, however, a rapid rise in energy expenditure in response to administration of a TGR5 agonist has not been described. Instead, the chronic thermogenic activity of TGR5 agonists has been ascribed to increased expression of type 2 iodothyronine deiodinase (D2), which converts thyroxine to triiodothyronine, because the TGR5 agonist cholic acid did not increase diet-induced thermogenesis in mice that lack D2. This is not a strong argument because β_3-adrenoceptor agonists also have little thermogenic activity in the absence of a functional thyroid system (Rubio et al., 1995; Golozoubova et al., 2004).

Activation of AMP-activated protein kinase (AMPK) in peripheral tissues promotes metabolic pathways that result in ATP production, whilst inhibiting those that require ATP utilization. Consistent with this role, nootkatone, a constituent of grapefruit that appears to activate kinases that are upstream of AMPK, elicited a rapid increase in energy expenditure in mice that was not associated with increased locomotor activity (Murase et al., 2010). Similar acute effects have not been described for directly acting AMPK activators, such as 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) or the thienopyridone A769662; nor for metformin, which activates AMPK by raising the tissue AMP concentration (Hardie, 2008). However, both A769662 and metformin reduced the respiratory exchange ratio (RER) of rats for about 3 h, after which the ratio increased for about 3 h (Cool et al., 2006), suggesting depletion of fat due to its increased oxidation or decreased synthesis. Metformin may cause a slight reduction in body weight in humans (Golay, 2008), but this seems to be due to decreased energy intake rather than increased energy expenditure. As in rats, however, metformin causes a transient reduction in RER (Arch, 2011). By comparison with other activators of AMPK, the rapid response to nootkatone is so unusual that it would be logical to check whether it might be a consequence of sympathetic activation.

Thyroid hormones and possibly fibroblast growth factor 21 (FGF21) are examples of treatments that bridge the gap between sympathomimetic compounds, which elicit rapid increases in energy expenditure, and some of the compounds and genetic modifications that are described in section Detection of Non-Acute Thermogenesis. In both cases, increased energy expenditure has been dissociated from any discernible effect on body weight, so it is unlikely that the increase in energy expenditure was a consequence of a change in body composition.

Depending on the dose, the onset of the response to thyroid hormones has been reported as 18 h to more than 5 days (Myant and Witney, 1967; de Lange et al., 2001; Kong et al., 2004). A variety of mechanisms, involving both decreased efficiency of ATP production and increased ATP utilization, for example activation sarcoendoplasmic reticulum Ca^{2+} ATPase (Silva, 2006; Ukropec et al., 2006b), have been suggested to explain thyroid-hormone induced thermogenesis, but it should also be remembered that thyroid hormones increase thermogenic responsiveness to the sympathetic nervous system (Ribeiro et al., 2001, 2010), once again suggesting that the sympathetic nervous system may play a role in thermogenesis.

FGF21 is released from liver and from brown and white adipocytes (Muise et al., 2008). Increased energy expenditure per animal was detected 2 days after intraperitoneal administration of FGF21 to diet-induced mice (Xu et al., 2009). The thermogenic effect of exogenous FGF21 may be a consequence of activation of brown adipose tissue, the induction of genes involved in oxidative metabolism in liver and adipose tissue, and the conversion of white adipocytes to cells that have some of the characteristics of brown adipocytes (Xu et al., 2009; Hondares et al., 2010; Fisher et al., 2012). The development of FGF21 itself as a drug presents significant challenges but recently workers from Genentech have described monoclonal antibodies that activate the FGF receptor 1 and have an antidiabetic effect in mice and monkeys (Wu et al., 2011; Foltz et al., 2012).

Obviously, compounds may elicit rapid rises in energy expenditure by promoting motor activity. Speakman (2013) discusses methods for measuring resting energy expenditure (REE), which should allow such a mechanism to be excluded.

TECHNICAL ISSUES

The evidence that the compounds described above elicit a rapid rise in energy expenditure has mostly been obtained using open-circuit indirect calorimetry. This involves passing air through a respiratory chamber and comparing its oxygen concentration after leaving the chamber with that of air entering the chamber (or leaving a chamber that contains no animals). The difference in these oxygen concentrations, coupled with the rate flow of air exiting the chamber cannot be used to measure oxygen consumption accurately because the flow of air into the chamber equals the flow out only when the RER = 1 (carbohydrate oxidation). When the RER is 0.72 (fat oxidation), oxygen consumption is underestimated by 6% if the two flow rates are assumed to be equal, because flow of air and therefore the amount of oxygen entering the chamber is higher than assumed. Fortunately, for the same consumption of oxygen, fat provides 6% less energy that carbohydrate. Consequently, calculation of energy expenditure assuming that only carbohydrate is being oxidized provides a very accurate measure of energy expenditure, whatever the balance of fuels. In other words, thanks to a mathematical fluke, measurement of oxygen concentration alone gives a more accurate measure of energy expenditure than of oxygen consumption. If the carbon dioxide concentration of the air leaving the chamber is also measured, it is possible to determine both carbon dioxide production and oxygen consumption and therefore the RER (Arch et al., 2006).

Changes in energy expenditure in response to specific compounds may be more rapid than they appear to be in publications. First, it takes a short time for changes in tissue oxygen utilization and carbon dioxide production to be reflected in expired air and then for any of that air to reach the gas analyzers. More importantly, most authors do not correct their calculation of energy expenditure to take account of the time that it takes for a change in energy expenditure to be fully reflected in the composition of the gases in the respiratory chamber. They ignore the fact that the volume of oxygen consumed is not only the difference between that entering and leaving the chamber: the decrease or increase in the amount of oxygen in the chamber must also be taken into

account. Similarly, carbon dioxide production is not just the difference between the amount leaving and entering the chamber. Assuming perfect mixing of the gases in the respiratory chamber, the half-life of the approach to the situation where the amount of oxygen in the chamber is constant after a step change in energy expenditure is 0.693 × chamber volume/flow rates. It is possible to calculate instantaneous energy expenditure by applying a correction derived from the rate of change of the difference between the oxygen concentration of air entering and leaving the chamber, but this can only be accurate if each chamber is monitored continuously (Arch et al., 2006; Speakman, 2013).

In our case, we keep animals in their home cages during the measurement of energy expenditure. If the chamber volume is 20 l and the flow rate is 0.5 l/min, the half-life of the approach to steady state is 28 min—far too long to compare energy expenditure with instantaneous measures of physical activity. Moreover, we do not monitor each chamber continuously. Others use smaller chamber volumes, so this is not so much of an issue. Nevertheless, it would be helpful if authors would describe both the chamber volume and the flow rate, and state whether their calculations of energy expenditure are corrected for the steady state issue.

SURROGATE MARKERS OF THERMOGENESIS

Not everybody has access to indirect calorimetry. This raises the question of what measurements might be used as surrogate markers of thermogenesis. Nothing can substitute for calorimetry as a quantitative measure of thermogenesis, but an elevation of the temperature of the heat-producing tissue, ideally relative to core temperature (Wellman and Marmon, 1985; Yoshioka et al., 1990), is good indication that thermogenesis is taking place. Occasionally, brown adipose tissue temperature alone is measured (Ueta et al., 2012), but measurement of core temperature alone, being technically easier, is far more common (Malinowska and Schlicker, 1997; Connoley et al., 1999; Luheshi et al., 1999; Russell and Tisdale, 2012a). Measurement of core temperature does not make the assumption that brown adipose tissue is the site of thermogenesis, but it ignores the possibility that there is a centrally-mediated increase in the set point of body temperature, as occurs in fever. An increase in body temperature due to a centrally-mediated increase in the set point of body temperature might be achieved by means of decreased heat loss rather than increased energy expenditure. Decreased heat loss becomes more important at higher ambient temperatures because it is at these temperatures that skin blood flow and evaporative water loss come into play as cooling mechanisms (Gordon, 2012).

Some workers have combined measurement of core temperature with exposure of animals to cold (typically 4–10°C). This approach has helped identify targets for thermogenic drugs in both brown adipose tissue and muscle (Bal et al., 2012; Fournier et al., 2012). Another surrogate is increased sympathetic activity, especially in brown adipose tissue, which has already been mentioned as an effect of bone morphogenetic protein 8B (Whittle et al., 2012).

Increased expression of the UCP1 gene, which may be detectable at the protein level within a day (Klein et al., 2000) and much earlier at the mRNA level, has been used by many workers as evidence that brown adipose tissue thermogenesis has been activated. A note of caution, however: peroxisome proliferator-activated receptor-γ agonists increase the expression of UCP-1 in brown adipose tissue, but they do not increase thermogenesis because sympathetic activity is reduced (Festuccia et al., 2008). Increased expression of UCP-1 is not a reliable indicator of thermogenesis, though it may be a consequence of UCP-1 activation (Ricquier et al., 2000). Increased binding of GDP to mitochondria isolated from brown adipose tissue is a better indicator of UCP-1 activation (Trayhurn and Milner, 1989) activation; this technique also seems to work for skeletal muscle mitochondria (Yoshida et al., 1998).

DETECTION OF RAPID RESPONSES TO GENETIC MODIFICATION

Detection of the acute effects of genetic manipulations on energy expenditure presents a significant challenge. It may be possible to induce genetic manipulation sufficiently rapidly, using for example a tetracycline-inducible site-specific recombinase system such as Cre-*loxP*, for the effect on energy expenditure to be assessed before there is a significant change in body composition (Zhang et al., 2012a). Energy expenditure does not appear to have been measured in any such animal, however. Another possible approach involves the use of adenoviral vectors. These have been used to express a protein that is cleaved to the myokine irisin in skeletal muscle, and irisin was claimed to increase energy expenditure (Bostrom et al., 2012). However, it was unclear how rapidly this effect developed, or whether energy expenditure was expressed per animal or relative to body weight. In another study, adeno-associated viral vectors were used to delete the hypothalamic CB1-R. Unfortunately energy expenditure was not measured until long after the body weights of the control and treated mice had diverged (Cardinal et al., 2012). Therefore, the methods used to assess whether genetic modification of a potential drug target affects energy expenditure are limited to non-acute thermogenesis, as described below.

DETECTION OF NON-ACUTE THERMOGENESIS

CORRECTION FOR DIFFERENCES IN BODY COMPOSITION

It should be more difficult to argue that a compound stimulates thermogenesis if its effect is not immediate or at least fails to appear within a day or two. After this time, energy expenditure might be affected by altered body weight or body composition. Compounds are more likely to affect lean body mass if animals are young (Rothwell and Stock, 1988; Arch et al., 1991) but few studies are conducted in older rodents. If a drug reduces body weight, especially lean body mass, this may mask its thermogenic effect. Studies in genetically modified animals present the same problems. Nevertheless, it is almost routine to read that lean animals have a higher energy expenditure than their more obese counterparts. The device used to justify such claims is to express energy expenditure relative to body weight or body weight$^{0.75}$. In the great majority of cases, the researchers then find that the leaner animals have the higher, mass-specific energy expenditure (Tschop et al., 2012). Those who study energy expenditure in humans have long-recognized that to understand the role of energy expenditure in the aetiology of obesity requires a more

sophisticated approach than this. If rodent data are to be treated differently from human data there needs to be a justification, but no such justification has been provided.

Prediction of energy expenditure in humans

The illogicality of dividing energy expenditure in rodents by body weight or body weight$^{0.75}$ in order to compare obese with lean rodents and why this can lead to erroneous conclusions has been discussed on a number of occasions (Arch et al., 2006; Butler and Kozak, 2010; Kaiyala et al., 2010; Cannon and Nedergaard, 2011; Even and Nadkarni, 2012; Tschop et al., 2012), including an article in this issue (Speakman, 2013). We shall not reproduce all these arguments but wish to demonstrate its absurdity by applying it to data for humans.

Equation 1 shows the correlation between 24-h energy expenditure (24EE; kcal) and body weight (BW in kg) in 177 male and female subjects with percentage body fat ranging from 3 to 50% (Ravussin et al., 1986).

$$24\text{EE} = 1043 + 13.0\text{BW} \quad (1)$$

The equation predicts that subjects that weigh 70 kg and 100 kg (which equate to body mass indices of 22.8 and 32.7 for a height of 1.75 m) will have 24-h energy expenditures of 1953 and 2343 kcal respectively—a 20% higher value in the heavier (and obese) subject. If 24-h EE is divided by body weight, however, as is common practice for rodent energy expenditure, we get 27.90 and 23.43 kcal/kg for the 70 and 100 kg subjects respectively—a 16% *lower* value in the heavier and more obese subject.

Similar predictions result from equations for REE, thereby excluding the possibility that differences in energy expenditure between lean and obese subjects are due to differences in locomotor activity.

$$\text{REE (kcal/d)} = 879 + 10.2\text{BW} \quad (2)$$

For example, Equation 2 (Owen et al., 1987) predicts REE values of 1593 and 1899 kcal/d for 70 and 100 kg men respectively—a 19% higher value in the heavier subject. By contrast, the values are 22.8 kcal/kg/d and 18.99 kcal/kg/d for the 70 and 100 kg men respectively—a 17% *lower* value in the heavier subject. If REE is expressed relative to body weight$^{0.75}$, the value is 9% lower in the heavier subject.

There is some evidence that obese subjects have a lower energy expenditure than lean subjects, but no workers have claimed such marked differences. Moreover, they have not used the simplistic approach of dividing energy expenditure by body weight but have adjusted their data for differences in body composition (Ravussin et al., 1988; Major et al., 2007). The reason why energy expenditure per kg body weight is lower in obese subjects is that fat mass has less influence than fat-free mass (FFM) on energy expenditure. Note that any influence that fat mass has on energy expenditure is little to do with the energy required for triglyceride turnover or even the energy requirements of white adipose tissue in which the triglyceride is stored, but may be mainly a consequence of the effects of adipokines, notably leptin, on energy utilization in other tissues (Even et al., 2001; Kaiyala et al., 2010; Wang et al., 2010b; Kaiyala and Schwartz, 2011).

Equations that relate either total or REE to body composition (FFM and %fat) demonstrate the greater influence of FFM compared to fat mass on energy expenditure:

$$24\text{EE} = 488 + 25.8\text{FFM} + 4.8\%\ \text{fat} \quad (3)$$

$$\text{REE (kcal/d)} = 560 + 5.39\text{BW} + 14.14\text{FFM} \quad (4)$$

Equation 3 (Ravussin et al., 1986) predicts that a subject weighing 70 kg of which 10 kg is fat will have a 24-h energy expenditure of 2104 kg, whereas a subject weighing 100 kg in whom all the extra weight is fat will have a 24-h energy expenditure of 2228 kcal—a difference of 124 kcal. On the other hand a muscular 100 kg subject in which the extra 30 kg is FFM (in total 90 kg FFM; 10 kg fat) will have a 24-h energy expenditure of 2858 kcal—a difference of 754 kcal from the 70 kg subject. Thus, extra lean tissue has 754/124 = 6.1 times the effect of extra fat on 24-h energy expenditure.

Equation 4 (Horie et al., 2011) predicts differences of 162 and 585 kcal depending on whether the extra tissue in the 100 kg person is due to extra fat or FFM—a 3.6-fold greater contribution from FFM.

The ratio of 6:1 for the relative contributions of equal weights of FFM and fat to 24-h energy expenditure is within the range of 5.0–6.7 calculated from experiments in mice for REE (Speakman and Johnson, 2000; Selman et al., 2001). Others have reported a ratio as low as 2 for total energy expenditure, which is below the ratio of 3.6 predicted by Equation 4 for REE in humans. The Lep^{ob}/Lep^{ob} mouse was an exception: fat mass was not a significant independent determinant of energy expenditure unless Lep^{ob}/Lep^{ob} mice were injected with leptin, its effect apparently being to increase the metabolic energy cost of lean body mass (Kaiyala et al., 2010; Kaiyala and Schwartz, 2011).

Analysis of rodent data

Speakman and other authors have argued that regression models should be used to compare energy expenditure data between lean and obese rodents (Arch et al., 2006; Kaiyala and Schwartz, 2011; Tschop et al., 2012; Speakman, 2013). The simplest regression method is to use analysis of covariance (ANCOVA) to relate energy expenditure to body weight for the whole data set—both control and treated or genetically altered groups. Energy expenditure values are shifted in parallel with the slope of the regression line to the mean body weight for the whole data set and then compared between groups (Speakman, 2013). More sophisticated methods involve multiple regression, for example separating out fat mass and lean body mass. It may be even better to separate out components of lean body mass, especially skeletal muscle mass, because this makes a relatively low contribution per unit mass to resting metabolic rate (Even et al., 2001). However, the more components that are included in the analysis, the lower its power to detect differences (Even and Nadkarni, 2012).

Regression analysis is undoubtedly more rigorous than division of energy expenditure by body weight, body weight$^{0.75}$ or even lean body mass. It shows whether differences in energy expenditure might be explained by the effect of a treatment or

genetic manipulation on body size or composition ("repartitioning," which is discussed further below). It may not always be possible to obtain information on body composition, however, especially components of lean body mass, during the in-life phase of a study. Other problems for some data sets are discussed in detail by Speakman (2013). One is that weights used for the regression analysis may not overlap sufficiently between treatment groups or genotypes. Consequently, the regression line may simply join the mean energy expenditure values for the two data sets, so that at each end there are equal numbers of points above the regression line. This inevitably leads to the conclusion that the data set belong to the same regression line. A wider range of weights might give a steeper slope with energy expenditure values for animals from the leaner group tending to lie above the line and those for the fatter group below the line. This problem is more likely to occur with small data sets.

A possible solution might be to fix the slope of the regression line using information for a larger number of animals of the same control and test types, rather than using data from the smaller experiment being analyzed. This would require that the slopes are similar for animals of the types being compared. Biologists take such an approach routinely when they use parametric statistics to compare data sets where $n < 5$, because it is only when $n \geq 5$ that it can be shown with reasonable power using Bartlett's test whether data have similar variances (exhibit homoscedasticity). Fortunately, we are allowed to use parametric statistics if previous work shows that similar data sets have similar variances (possible after transformation). Without this rule, we could never say that the data sets 1, 2, 3, and 1001, 1002, 1003 are significantly different because the non-parametric Mann–Whitney two-sided U-test gives $P = 0.1$.

Even if body weights or the weights of body components overlap, small data sets increase the probability that the regression line will not be significant, especially if weights of individuals are similar in each group. It has been argued that in such cases it is acceptable to divide energy expenditure by lean body mass because it contributes more than fat mass to whole body energy expenditure (Butler and Kozak, 2010). Fat mass cannot be ignored, however, and Speakman (2013) argues that dividing energy expenditure by lean body mass does not increase the power to separate data sets.

This raises the point that the great majority of studies on the effect of genetics on energy expenditure have been underpowered (Tschop et al., 2012; Speakman, 2013). Some workers have gone so far as to suggest that indirect calorimetry is not sufficiently accurate to be of value in long term studies of energy balance (Even and Nadkarni, 2012). Whilst true in many cases, there may be a few mechanisms, such as those that impact on sympathetic activity or the response of brown adipose tissue to sympathetic activity, that have sufficiently large effects for differences in energy expenditure to be detected. For example, in our hands the *Lep*^{ob}/*Lep*^{ob} mouse had a lower energy expenditure per animal than wild-type mice, but only at a temperature below thermoneutrality (Trayhurn and James, 1978; Wilson et al., 1984). In addition, pair-feeding studies in which young *Lep*^{ob}/*Lep*^{ob} mice were yoked to the *ad libitum* energy intake of their lean siblings became obese, reflecting a lower level of energy expenditure, and these differences were much less marked at thermoneutrality than at lower environmental temperatures (Thurlby and Trayhurn, 1979). This phenotype may be due to the *Lep*^{ob}/*Lep*^{ob} mouse not raising sympathetic activity adequately in response to cold (Reichling et al., 1988). [Others have found that *Lep*^{ob}/*Lep*^{ob} mice have a higher energy expenditure than their wild type controls possibly because they measured energy expenditure under different conditions (Himms-Hagen, 1997; Butler and Kozak, 2010)].

The *Lep*^{ob}/*Lep*^{ob} mouse is of course an example of an obese mutant, whereas the focus of this article is leanness. It is therefore disappointing that energy expenditure has not been measured in "skinny" mice, which overexpress leptin, although increased insulin sensitivity has been described (Ogawa et al., 1999). Mice in which the soluble leptin receptor was overexpressed had increased energy expenditure relative to lean body mass, but they also had lower amounts of both lean and fat mass, making interpretation of this finding difficult for reasons explained above (Lou et al., 2010).

In those cases where the data are not suitable for analysis using regression models, the best solution may be to adjust energy expenditure data according to the size of key organs using factors described in the literature (Elia, 1992; Even et al., 2001; Wang et al., 2010a,b). An interesting suggestion is that energy expenditure for mice of differing adiposities might be divided by lean body mass plus 0.2 × fat mass (Even and Nadkarni, 2012). This is consistent with one of the reports discussed above (Selman et al., 2001), which suggested that, relative to its weight lean, body mass contributes five times more than fat mass to energy expenditure. This approach is not ideal, however, and must not be used thoughtlessly, especially in animals that lack a functional leptin system (Kaiyala et al., 2010) As mentioned in section Prediction of Energy Expenditure in Humans, the influence that adipose tissue has on energy expenditure may be mainly a consequence of the effects of adipokines on energy expenditure in other tissues, so the consequences of manipulating adipokine secretion must be considered.

NON-ACUTE STIMULATION OF ENERGY EXPENDITURE PER ANIMAL

Repartitioning

There is little difficulty in arguing that a compound is thermogenic, or a genetically modified mouse suggests a target for thermogenic drugs if energy expenditure *per animal* is increased, whilst body fat is decreased (or at least unchanged) and lean tissue is not increased. A compound that increases energy expenditure per animal because it increases the proportion of more metabolically active organs, such as liver or brain, relative to skeletal muscle might be better described as a repartitioning or anabolic agent, rather than a thermogenic drug. A possible example from our own work is the 11β-hydroxysteroid dehydrogenase type 1 inhibitor BVT.2733, which increased both lean body mass and energy expenditure (Wang et al., 2006). The comparison in this example was with pair-fed mice because BVT.2733 reduced food intake and so its effect was to prevent the loss of lean tissue in response to reduced food intake.

Genetic modifications that increase energy expenditure per animal because they alter body composition may suggest targets

for repartitioning agents. Thus, in our own work, both lean body mass and energy expenditure were higher in male acyl CoA:diacylglycerol acyltransferase 1-null than in wild-type mice when the mice were fed on a high fat diet (Wang et al., 2007). Some compounds or genetic modifications may increase energy expenditure due to both a repartitioning effect and a thermogenic effect that is independent of the repartitioning effect. For example, the β_2-adrenoceptor agonist clenbuterol may cause thermogenesis in obese (*fa/fa*) rats by stimulating brown adipose tissue and by repartitioning energy to skeletal muscle (Rothwell and Stock, 1987).

Non-acute thermogenesis in response to compounds

β_3-Adrenoceptor agonists and FGF21 are examples of treatments that increase energy expenditure per animal when they are given for a week or more, even though body fat content is by this time decreased and body lean content is not increased (Arch and Ainsworth, 1983a; Xu et al., 2009). Leptin may also have increased energy expenditure per animal when given for more than a week to rat pups, though data were expressed relative to body weight, which may have decreased slightly (Stehling et al., 1996). In adult mice, leptin had a more subtle effect that developed over days: it reduced the daily minima of energy expenditure when the mice were food restricted (Doring et al., 1998). Chemical sympathectomy reduced weight loss in response to intracerebroventricular infusion of leptin by 60% over 10 days in rats, despite it not affecting the response of food intake to leptin (Dobbins et al., 2003). This suggests that weight loss was partly due to thermogenesis driven by the sympathetic nervous system. It is consistent with the thermogenic effect of leptin on days 2–6 being reduced by about 50% in mice that lack all three β-adrenoceptors (see section Acute Stimulation of Energy Expenditure). However, the same paper reports that leptin did not cause weight loss over 6 days in either wild type or "β-less" mice other than by reducing food intake (Asensio et al., 2008).

The examples discussed so far are all treatments that also increase energy expenditure almost immediately (β_3-adrenoceptor agonists) or at least within 2 days (FGF21; leptin). It is much more difficult to find examples of compounds that increase energy expenditure per animal after more than 2 days but not earlier (without increasing lean body mass). B- and C-type natriuretic peptides (BNP and CNP), which activate receptors that are linked to guanylyl cyclases, seem to promote thermogenesis in brown adipose tissue. BNP increased oxygen consumption per mouse after it had been infused for 7 days but its acute effect was not reported (Inuzuka et al., 2010; Bordicchia et al., 2012).

Most claims of increased energy expenditure in lean animals are shown to be ill-founded on detailed examination. For example, antagonism of the activin receptor IIB by immunological means has been claimed to increase energy expenditure either by increasing muscle mass or by increasing brown adipocyte adipogenesis (Fournier et al., 2012; Koncarevic et al., 2012). Energy expenditure was measured after 28 or 60 days, however, and expressed relative to body weight, which in at least one of these studies (Koncarevic et al., 2012) was decreased in proportion to the increase in energy expenditure per gram body weight. Another recent example is JD5037, which is a CB1-R inverse agonist that has poor brain penetration. JD5037 reduced food intake and body weight in diet-induced obese mice (Tam et al., 2012). It was claimed that it increased energy expenditure, but this was expressed relative to body weight$^{0.75}$ and it can be calculated that the ratio of the oxygen consumption values in treated and control mice on day 21 of treatment is very similar to the inverse of the ratio of the body weight$^{0.75}$ values at that time. In other words, energy expenditure per mouse was not altered by treatment. JD5037 affects body weight by reversing leptin resistance and leptin increases sympathetic activity, so it would not have been surprising if it had increased energy expenditure per mouse. However, JD5037 reverses leptin resistance by reversing hyperleptinaemia, which would tend to lower sympathetic activity. Thus, the lower concentration of leptin and the increased sensitivity to leptin may have had roughly equal and opposing effects on energy expenditure. [It is worth noting that leptin expression and release is inhibited by the sympathetic nervous system, so leptin production as well as responsiveness to leptin is subject to feedback control (Mantzoros et al., 1996; Trayhurn et al., 1996)].

The AMPK activator AICAR increased energy expenditure per animal after 4 and 8 weeks' administration to rats compared to pair-fed control (Gaidhu et al., 2011). Energy expenditure was measured weekly and there is no evidence given in this paper or elsewhere of it having an earlier or acute thermogenic effect (although an acute lowering of RER was described in section Acute Stimulation of Energy Expenditure). Thus, AICAR may be an example of a treatment whose thermogenic effect takes more than a week to develop. AICAR also reduced fat pad weights compared to the pair-fed controls, which suggests that weight loss may have been due to thermogenesis; but if so it is surprising that fat mass increased at the same rate as in controls between 4 and 8 weeks of treatment even though thermogenesis did not diminish. The effect of AICAR was not that expected for a peripherally acting AMPK activator because energy expenditure increased only during the dark period and was associated with increased locomotor activity. It is well-established that activation of AMPK in the hypothalamus increases food intake, which raises the possibility that increased locomotor activity elicited by AICAR may have been due to enhancement of food seeking behavior. In the light of this discussion the conclusions of a recent review are of interest. These were that AMPK is "always activated by mitochondrial uncoupling" mediated by UCP-1, and may augment the effect of uncoupling but "activation of AMPK alone does not lead directly to an induction of energy expenditure (Klaus et al., 2012)."

Non-acute thermogenesis in response to genetic modifications

The UCP-1 knockout mouse is an example of a genetic modification that elicits (paradoxically) a detectable increase in energy expenditure per animal. This difference could not be detected until the ambient temperature had fallen to about 10–12°C, however. It seemed to be due to the mouse being forced to use a mechanism independent of UCP-1 to generate heat. This mechanism must be less efficient than UCP-1 activation in maintaining body temperature (Ukropec et al., 2006a). Another example is the melanin-concentrating hormone 1 receptor knockout mouse,

but in this case the increase was associated with increased locomotor activity (Marsh et al., 2002). The renin knockout mouse displayed an increased energy expenditure relative to its lean body mass, and at least in the case of REE, it seems likely that energy expenditure per mouse was raised (Takahashi et al., 2007).

These are rare examples. An example that does not stand up to scrutiny is that overexpression of FGF19 increases energy expenditure (Tomlinson et al., 2002). It was only energy expenditure expressed relative to body weight$^{0.75}$ that was increased. Once again it can be calculated from the data presented that energy expenditure per animal was no different between transgenic and wild-type mice. The authors also argued that energy expenditure must have been increased because food intake was higher in the transgenic mice, but food intake was expressed relative to body weight (not like energy expenditure as body weight$^{0.75}$) and it was not increased on a whole animal basis.

We have already argued that exogenous FGF21 has a thermogenic effect. This was detectable after 19 days of treatment (Xu et al., 2009). It is perhaps therefore surprising that energy expenditure expressed relative to body weight was similar in mice in which FGF21 was overexpressed and wild-type mice, even though the FGF21 overexpressing mice were much smaller. Thus, energy expenditure per animal was lower in the transgenic mice in proportion to their weight. However, the body composition of the transgenic and wild-type mice was similar, so this is not a comparison between a lean and an obese strain. It appears instead to be a situation where energy expenditure really does reflect body size (Zhang et al., 2012b).

There are a few examples of where the correlation between energy expenditure and body weight has been shown to be altered by genetic modification. Some of these involve modifications that affect the development and function of brown adipose tissue. Thus, ANCOVA showed that energy expenditure at a given body weight was increased in mice with a null mutation for transient receptor potential vanilloid 4 (TRPV4). An antagonist of TRPV4 increased the expression of UCP-1 in brown adipose tissue, but its effect on energy expenditure was not reported (Ye et al., 2012). Mice in which UCP-1 was overexpressed in skeletal muscle were leaner than wild-type mice (unlike the UCP-1 knockout mice, this is predictable rather than paradoxical). Mean energy expenditure was not increased, but for a given body weight it was higher in the genetically modified animals (Couplan et al., 2002). It might be argued that this was because the weights of the brain and liver—organs that make a disproportionate contribution to energy expenditure—were increased. However, other work showed that energy expenditure (which was expressed relative to body weight) was increased at night but not during the day, despite there being no difference in locomotor activity between the wild-type and transgenic mice. Daytime energy expenditure was, therefore in effect, a within-animal control for night-time energy expenditure. The authors therefore concluded that muscle energetic efficiency was decreased by over-expression of UCP-1 in skeletal muscle (Klaus et al., 2005). The fact that activation of UCP-1 is the mechanism of thermogenesis in brown adipose tissue seems to support the view that this is a genuine example of a genetic modification that stimulates thermogenesis, but as mentioned previously, the expression of UCP-1 indicates only the capacity for thermogenesis in brown adipose tissue and not whether this capacity is being used. Other examples of the effect of overexpression of UCP-1 in skeletal muscle, white adipose tissue and liver have been reviewed recently in relation to the role of AMPK in mediating the phenotype (Klaus et al., 2012).

INDIRECT EVIDENCE

It may be possible to deduce that a compound or genetic modification has increased energy expenditure without directly measuring expenditure itself. One such situation is where energy intake is increased and yet the treated or genetically modified animal has a reduced body fat content and no increase in lean body mass. For example, repeated administration of the β_3-adrenoceptor agonist BRL26830 increased food intake in lean mice but reduced their body lipid content and had no effect on lean body mass (Arch and Ainsworth, 1983b). In this instance it was also apparent from indirect calorimetry that energy expenditure per animal was elevated. Similarly, 11β-hydroxysteroid dehydrogenase type 1 knockout mice fed on a high fat diet were less obese than wild-type mice, despite having a higher caloric intake (Morton et al., 2004). Their increased core temperature also suggested that energy expenditure was raised. By contrast, increased energy expenditure per animal was not evident in mice that lacked acetyl-CoA carboxylase-2, but it appeared that energy expenditure must have been increased because the knockout mice consumed more energy than the wild-type mice but had a reduced body fat and lean content (Abu-Elheiga et al., 2001; Choi et al., 2007). The knockout mice showed increased energy expenditure relative to lean body mass, but it can be calculated from the data provided that energy expenditure per animal was no different between the knockout and control mice (Choi et al., 2007). It is difficult to understand how energy intake per mouse could have been so much higher (20–30%) in the knockout than the wild-type mice and yet energy expenditure per mouse was not increased. Other workers have failed to replicate these findings (Hoehn et al., 2010; Olson et al., 2010). Nevertheless, the principle that it may be possible to deduce from energy intake and body composition that energy expenditure has increased remains valid. This assumes, of course, that the food eaten is absorbed normally and energy is not lost as a result of glycosuria or ketosis; in other words, that the metabolisability of the diet is not altered.

Another situation that might suggest increased thermogenesis is where body fat content is reduced, but there is no decrease in food intake, or food intake is kept equal between groups by pair-feeding. The problem with this argument is that it is easier to show that body fat content differs between two groups of animals than that their energy intake (especially energy absorption) differs. Without positive evidence of increased energy expenditure such arguments are usually unreliable. A similar argument is that drugs that are both acutely thermogenic and acutely anorectic cause fat loss in the longer term through thermogenesis, because the effect on food intake usually wanes rapidly and food intake over the whole course of the study is not reduced (Arch, 1981; Day and Bailey, 1998; Picard et al., 2000; Billes and Cowley, 2008; Herling et al., 2008). This logic is of little value, however, unless it

is demonstrated that the thermogenic effect of the treatment does not disappear in the same way that the anorectic effect disappears.

One instance where the thermogenic effect of treatment did not disappear was when the *Lep*^{ob}/*Lep*^{ob} mouse was dosed repeatedly with a β_3-adrenoceptor agonist (Arch and Ainsworth, 1983a). The *Lep*^{ob}/*Lep*^{ob} mouse is amenable to such a finding because it has low sympathetic activity at temperatures below thermoneutrality and it has a lot of fat to lose. Their lean littermates do not have as much fat in their whole body as the *Lep*^{ob}/*Lep*^{ob} mouse loses in response to treatment with β_3-adrenoceptor agonists for a month. Consequently, since lean body mass is not reduced, lean mice must either have a reduced thermogenic response as treatment continues or they must increase their energy intake (Arch and Ainsworth, 1983a). Unfortunately, the *Lep*^{ob}/*Lep*^{ob} mouse is not a good general model of human obesity because it lacks leptin, and this has many downstream consequences—low sympathetic activity and high hypothalamic neuropeptide Y release, for example. Thus, any intervention that corrects leptin deficiency or any of its downstream consequences is likely to have an exaggerated effect in the *Lep*^{ob}/*Lep*^{ob} mouse.

Full carcass analysis coupled with careful measurement of metabolisable energy intake is occasionally used to calculate energy expenditure (Dulloo and Miller, 1987; Mercer and Trayhurn, 1987). Ideally, such studies should be conducted using metabolic chambers and faecal and urinary energy should be measured. This approach has been used to demonstrate that the β_2-adrenoceptor agonist clenbuterol increases thermogenesis as well as increasing lean tissue at the expense of fat (Rothwell and Stock, 1987).

TRANSLATION OF RODENT FINDINGS TO HUMANS

It is important to understand how, and when (i.e., under what conditions), a compound increases energy expenditure in rodents before investigating whether it does the same in humans. Similarly, it is sensible to know how a genetic modification increases energy expenditure before seeking drugs that act via the product of the modified gene. An obvious example of the "how" issue is where thermogenesis is associated with increased locomotor activity. Whether this is an acceptable mechanism of action for an antiobesity drug is debatable, but it clearly would make no sense to expect energy expenditure to be raised in humans when they are resting. Thyroid hormone-related mechanisms raise the spectre of cardiac stimulation, loss of skeletal muscle, bone wasting, fatigue, and CNS effects (Crunkhorn and Patti, 2008), although there has been interest in the possibility that selective activators of thyroid hormone receptor-β might avoid such problems (Ribeiro, 2008).

The significance of the "when" question can be illustrated by the influence of ambient temperature on the phenotype of the UCP-1 knockout mouse. The phenotype of these mice appears to depend on whether they are housed at thermoneutrality or below thermoneutrality. Reports vary, however, from the UCP-1 knockout mouse being obese and especially sensitive to diet-induced obesity when housed at thermoneutrality (Feldmann et al., 2009), to their being resistant to diet-induced obesity when housed at 20°C (Liu et al., 2003). Most humans live at temperatures near thermoneutrality, which suggests that, if anything, *activation* of UCP-1 in brown adipose tissue, rather than its absence should increase whole body thermogenesis. However, there is the possibility that less efficient thermogenic mechanisms in other tissues might shut down when brown adipose tissue is activated, entirely negating the effect of UCP-1 activation.

A common situation is that a compound increases energy expenditure because it increases the activity of the sympathetic nervous system. Activation of the sympathetic nervous system in humans has far less effect on energy expenditure in humans than in rodents. The maximum increase in energy expenditure in humans at thermoneutrality in response to sympathomimetic drugs is about 30% (Schiffelers et al., 2000, 2001), whereas in rodents increases of two- or threefold are possible if the animals are maintained at around 21°C (Wilson et al., 1984; Wernstedt et al., 2006; Feldmann et al., 2009). Even greater fold increases are possible if the animals are maintained at thermoneutrality to suppress their baseline sympathetic activity. This also applies to a centrally acting agent such as ephedrine: it should not be assumed that centrally acting drugs cannot raise sympathetic activity if rodents or humans are at thermoneutrality (Wilson et al., 1984; Gordon, 2012). It is important to remember that the thermoneutral zone may be lowered if animals are housed in groups so that they can huddle, or if they are given plenty of bedding to wrap up in or a wheel to keep active; this is reflected in the differences in thermogenic activity and capacity of their brown adipose tissue when mice are housed singly or in groups of varying size (Jennings et al., 1986). Large effects may also occur in *Lep*^{ob}/*Lep*^{ob} mice and similar models of leptin dysfunction, which have low sympathetic activity (Wilson et al., 1984). Indeed, it is possible that increased energy expenditure might not translate at all from such models to humans.

To give some perspective on thermogenesis in humans, a simple calculation shows that anorectic drugs must alter energy balance by about 10% to cause weight loss of about 5 kg over six months, which historically has been a typical effect (Rucker et al., 2007). It may be difficult to achieve this over 24 h if the maximum effect is 30%. On the other hand, despite their relatively low efficacy as stimulants of energy expenditure, sympathomimetic drugs, such as β-adrenoceptor agonists, have been shown to improve insulin sensitivity markedly in humans (Mitchell et al., 1989; Smith et al., 1990).

What if a compound does increase sympathetic activity in humans? Might it have undesirable side-effects due to generalized sympathetic activation? This was the case with sibutramine. It is unclear whether thermogenesis contributed to its antiobesity effect, but sympathetic activity contributed to the cardiovascular side effects that eventually led to its withdrawal (James et al., 2010). The thermogenic effect of rimonabant, by contrast, was not detected in humans, although another CB1-R antagonist did raise energy expenditure (Addy et al., 2008).

A drug that raises sympathetic activity selectively in brown adipose tissue, skeletal muscle or white adipose tissue, rather than in the cardiovascular system would be ideal, but is this feasible? Despite evidence that sympathetic nerves to brown adipose tissue can be activated selectively (Morrison, 2001; Kosari et al., 2011), leptin, the main role of which is to regulate energy

balance, raises sympathetic activity in "non-metabolic" tissues (Haynes et al., 1997; Rahmouni and Morgan, 2007). The current interest in drugs and drug targets that increase the response of brown adipose tissue to the sympathetic nervous system offers a possible way to avoid these problems.

CONCLUSION

As long as we seek drugs for the treatment of obesity, the questions, "Is it thermogenic?" and "Is it a target for thermogenic drugs?" will continue to be asked. It is a relatively simple matter to show whether a compound stimulates thermogenesis if energy expenditure increases rapidly and cannot be a consequence of altered body composition. The clearest examples of such compounds are those that activate or mimic the sympathetic nervous system, and so it is wise to check whether any compound whose effect appears rapidly acts via such a mechanism.

Even if a compound has an anti-obesity effect and is acutely thermogenic, it cannot be assumed that this is why it causes weight loss unless it raises 24-h energy expenditure throughout the weight loss experiment. This then raises an issue that also afflict studies in which potential drug targets are genetically modified in mice: how should energy expenditure be expressed. The wrong way, at least when comparing lean and obese mice, is to divide energy expenditure by body weight or body weight$^{0.75}$. The surest way is to express energy expenditure per mouse and then to check whether energy expenditure has increased because there is an increase in the absolute amount of lean tissue—in other words the treatment would be better described as "repartitioning" rather than thermogenic.

There are other ways of detecting thermogenesis than measuring energy expenditure, and there are ways of comparing energy expenditure measurements that allow for differences in body weight and composition, but they should not be used without an understanding of their limitations.

ACKNOWLEDGMENTS

We thank Professor Mike Cawthorne for his astute comments on the manuscript.

REFERENCES

Abu-Elheiga, L., Matzuk, M. M., Abo-Hashema, K. A., and Wakil, S. J. (2001). Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2. *Science* 291, 2613–2616.

Addy, C., Wright, H., Van Laere, K., Gantz, I., Erondu, N., Musser, B. J., et al. (2008). The acyclic CB1R inverse agonist taranabant mediates weight loss by increasing energy expenditure and decreasing caloric intake. *Cell Metab.* 7, 68–78.

Arch, J. R. (1981). The contribution of increased thermogenesis to the effect of anorectic drugs on body composition in mice. *Am. J. Clin. Nutr.* 34, 2763–2769.

Arch, J. R. (2011). Thermogenesis and related metabolic targets in anti-diabetic therapy. *Handb. Exp. Pharmacol.* 201–255.

Arch, J. R., and Ainsworth, A. T. (1983a). Thermogenic and antiobesity activity of a novel β-adrenoceptor agonist (BRL 26830A) in mice and rats. *Am. J. Clin. Nutr.* 38, 549–558.

Arch, J. R. S., and Ainsworth, A. T. (1983b). Reduction of obesity in mice with a novel type of thermogenic β-adrenergic agonist. *Int. J. Obes.* 7, 85–86.

Arch, J. R., Ainsworth, A. T., and Cawthorne, M. A. (1982). Thermogenic and anorectic effects of ephedrine and congeners in mice and rats. *Life Sci.* 30, 1817–1826.

Arch, J. R., Ainsworth, A. T., Cawthorne, M. A., Piercy, V., Sennitt, M. V., Thody, V. E., et al. (1984). Atypical β-adrenoceptor on brown adipocytes as target for anti-obesity drugs. *Nature* 309, 163–165.

Arch, J. R., Hislop, D., Wang, S. J. Y., and Speakman, J. R. (2006). Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals. *Int. J. Obes.* 30, 1322–1331.

Arch, J. R. S., Cawthorne, M. A., Coney, K. A., Gusterson, B. A., Piercy, V., Sennitt, M. V., et al. (1991). "β-adrenoceptor-mediated control of thermogenesis, body composition and glucose homeostasis," in *Obesity and Cachexia,* eds N. J. Rothwell and M. J. Stock (Chichester: Wiley), 241–268.

Arch, J. R. S., Piercy, V., Thurlby, P. L., Wilson, C., and Wilson, S. (1987). "Thermogenic and lipolytic drugs for the treatment of obesity: old ideas and new possibilities," in *Recent Advances in Obesity Research,* eds E. M. Berry, S. H. Blondheim, H. E. Eliahou, and E. Shafrir (London: John Libbey), 300–311.

Asensio, C. S., Arsenijevic, D., Lehr, L., Giacobino, J. P., Muzzin, P., and Rohner-Jeanrenaud, F. (2008). Effects of leptin on energy metabolism in β-less mice. *Int. J. Obes. (Lond.)* 32, 936–942.

Bal, N. C., Maurya, S. K., Sopariwala, D. H., Sahoo, S. K., Gupta, S. C., Shaikh, S. A., et al. (2012). Sarcolipin is a newly identified regulator of muscle-based thermogenesis in mammals. *Nat. Med.* 18, 1575–1579.

Billes, S. K., and Cowley, M. A. (2008). Catecholamine reuptake inhibition causes weight loss by increasing locomotor activity and thermogenesis. *Neuropsychopharmacology* 33, 1287–1297.

Bordicchia, M., Liu, D., Amri, E. Z., Ailhaud, G., Dessi-Fulgheri, P., Zhang, C., et al. (2012). Cardiac natriuretic peptides act via p38 MAPK to induce the brown fat thermogenic program in mouse and human adipocytes. *J. Clin. Invest.* 122, 1022–1036.

Bostrom, P., Wu, J., Jedrychowski, M. P., Korde, A., Ye, L., Lo, J. C., et al. (2012). A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature* 481, 463–468.

Brooks, B. J., Arch, J. R., and Newsholme, E. A. (1983). Effect of some hormones on the rate of the triacylglycerol/fatty-acid substrate cycle in adipose tissue of the mouse *in vivo*. *Biosci. Rep.* 3, 263–267.

Butler, A. A., and Kozak, L. P. (2010). A recurring problem with the analysis of energy expenditure in genetic models expressing lean and obese phenotypes. *Diabetes* 59, 323–329.

Cannon, B., and Nedergaard, J. (2011). Nonshivering thermogenesis and its adequate measurement in metabolic studies. *J. Exp. Biol.* 214, 242–253.

Cardinal, P., Bellocchio, L., Clark, S., Cannich, A., Klugmann, M., Lutz, B., et al. (2012). Hypothalamic CB1 cannabinoid receptors regulate energy balance in mice. *Endocrinology* 153, 4136–4143.

Challis, R. A., Arch, J. R., and Newsholme, E. A. (1985). The rate of substrate cycling between fructose 6-phosphate and fructose 1, 6-bisphosphate in skeletal muscle from cold-exposed, hyperthyroid or acutely exercised rats. *Biochem. J.* 231, 217–220.

Choi, C. S., Savage, D. B., Abu-Elheiga, L., Liu, Z. X., Kim, S., Kulkarni, A., et al. (2007). Continuous fat oxidation in acetyl-CoA carboxylase 2 knockout mice increases total energy expenditure, reduces fat mass, and improves insulin sensitivity. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16480–16485.

Choo, J. J. (2003). Green tea reduces body fat accretion caused by high-fat diet in rats through β-adrenoceptor activation of thermogenesis in brown adipose tissue. *J. Nutr. Biochem.* 14, 671–676.

Clapham, J. C., and Arch, J. R. (2007). Thermogenic and metabolic antiobesity drugs: rationale and opportunities. *Diabetes Obes. Metab.* 9, 259–275.

Collins, L. C., Walker, J., and Stamford, B. A. (1996a). Smoking multiple high- versus low-nicotine cigarettes: impact on resting energy expenditure. *Metabolism* 45, 923–926.

Collins, S., Kuhn, C. M., Petro, A. E., Swick, A. G., Chrunyk, B. A., and Surwit, R. S. (1996b). Role of leptin in fat regulation. *Nature* 380, 677.

Connoley, I. P., Liu, Y. L., Frost, I., Reckless, I. P., Heal, D. J., and Stock, M. J. (1999). Thermogenic effects of sibutramine and its metabolites. *Br. J. Pharmacol.* 126, 1487–1495.

Cool, B., Zinker, B., Chiou, W., Kifle, L., Cao, N., Perham, M., et al. (2006). Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell Metab.* 3, 403–416.

Couplan, E., Gelly, C., Goubern, M., Fleury, C., Quesson, B., Silberberg, M., et al. (2002). High level of uncoupling protein 1 expression in muscle of transgenic mice selectively affects muscles at rest and decreases their IIb fiber content. *J. Biol. Chem.* 277, 43079–43088.

Crunkhorn, S., and Patti, M. E. (2008). Links between thyroid hormone action, oxidative metabolism, and diabetes risk? *Thyroid* 18, 227–237.

Cypess, A. M., Chen, Y. C., Sze, C., Wang, K., English, J., Chan, O., et al. (2012). Cold but not sympathomimetics activates human brown adipose tissue *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 109, 10001–10005.

Day, C., and Bailey, C. J. (1998). Effect of the antiobesity agent sibutramine in obese-diabetic ob/ob mice. *Int. J. Obes. Relat. Metab. Disord.* 22, 619–623.

de Lange, P., Lanni, A., Beneduce, L., Moreno, M., Lombardi, A., Silvestri, E., et al. (2001). Uncoupling protein-3 is a molecular determinant for the regulation of resting metabolic rate by thyroid hormone. *Endocrinology* 142, 3414–3420.

Dobbins, R. L., Szczepaniak, L. S., Zhang, W., and McGarry, J. D. (2003). Chemical sympathectomy alters regulation of body weight during prolonged ICV leptin infusion. *Am. J. Physiol. Endocrinol. Metab.* 284, E778–E787.

Doring, H., Schwarzer, K., Nuessle in-Hildesheim, B., and Schmidt, I. (1998). Leptin selectively increases energy expenditure of food-restricted lean mice. *Int. J. Obes. Relat. Metab. Disord.* 22, 83–88.

Dulloo, A. G., and Miller, D. S. (1987). Screening of drugs for thermogenic anti-obesity properties: antidepressants. *Ann. Nutr. Metab.* 31, 69–80.

Dulloo, A. G., Seydoux, J., Girardier, L., Chantre, P., and Vandermander, J. (2000). Green tea and thermogenesis: interactions between catechin-polyphenols, caffeine and sympathetic activity. *Int. J. Obes. Relat. Metab. Disord.* 24, 252–258.

Dunbar, J. C., Hu, Y., and Lu, H. (1997). Intracerebroventricular leptin increases lumbar and renal sympathetic nerve activity and blood pressure in normal rats. *Diabetes* 46, 2040–2043.

Elia, M. (1992). "Organ and tissue contributions to metabolic rate," in *Energy Metabolism: Tissue Determinants and Cellular Corollaries,* eds J. M. Kinney and H. N. Tucker (New York, NY: Raven), 61–80.

Even, P. C., Rolland, V., Roseau, S., Bouthegourd, J. C., and Tome, D. (2001). Prediction of basal metabolism from organ size in the rat: relationship to strain, feeding, age, and obesity. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 280, R1887–R1896.

Even, P. C., and Nadkarni, N. A. (2012). Indirect calorimetry in laboratory mice and rats: principles, practical considerations, interpretation and perspectives. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 303, R459–R476.

Feldmann, H. M., Golozoubova, V., Cannon, B., and Nedergaard, J. (2009). UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell Metab.* 9, 203–209.

Festuccia, W. T., Oztezcan, S., Laplante, M., Berthiaume, M., Michel, C., Dohgu, S., et al. (2008). Peroxisome proliferator-activated receptor-γ-mediated positive energy balance in the rat is associated with reduced sympathetic drive to adipose tissues and thyroid status. *Endocrinology* 149, 2121–2130.

Fisher, F. M., Kleiner, S., Douris, N., Fox, E. C., Mepani, R. J., Verdeguer, F., et al. (2012). FGF21 regulates PGC-1α and browning of white adipose tissues in adaptive thermogenesis. *Genes Dev.* 26, 271–281.

Foltz, I. N., Hu, S., King, C., Wu, X., Yang, C., Wang, W., et al. (2012). Treating diabetes and obesity with an FGF21-mimetic antibody activating the βKlotho/FGFR1c receptor complex. *Sci. Transl. Med.* 4, 162ra153.

Fournier, B., Murray, B., Gutzwiller, S., Marcaletti, S., Marcellin, D., Bergling, S., et al. (2012). Blockade of the activin receptor IIb activates functional brown adipogenesis and thermogenesis by inducing mitochondrial oxidative metabolism. *Mol. Cell. Biol.* 32, 2871–2879.

Fruhbeck, G., Becerril, S., Sainz, N., Garrastachu, P., and Garcia-Velloso, M. J. (2009). BAT: a new target for human obesity? *Trends Pharmacol. Sci.* 30, 387–396.

Gaidhu, M. P., Frontini, A., Hung, S., Pistor, K., Cinti, S., and Ceddia, R. B. (2011). Chronic AMP-kinase activation with AICAR reduces adiposity by remodeling adipocyte metabolism and increasing leptin sensitivity. *J. Lipid Res.* 52, 1702–1711.

Gavrilova, O., Marcus-Samuels, B., and Reitman, M. L. (2000). Lack of responses to a β_3-adrenergic agonist in lipoatrophic A-ZIP/F-1 mice. *Diabetes* 49, 1910–1916.

Golay, A. (2008). Metformin and body weight. *Int. J. Obes. (Lond.)* 32, 61–72.

Golozoubova, V., Gullberg, H., Matthias, A., Cannon, B., Vennstrom, B., and Nedergaard, J. (2004). Depressed thermogenesis but competent brown adipose tissue recruitment in mice devoid of all hormone-binding thyroid hormone receptors. *Mol. Endocrinol.* 18, 384–401.

Gordon, C. J. (2012). Thermal physiology of laboratory mice: defining thermoneutrality. *J. Therm. Biol.* 37, 654–685.

Granneman, J. G., Burnazi, M., Zhu, Z., and Schwamb, L. A. (2003). White adipose tissue contributes to UCP1-independent thermogenesis. *Am. J. Physiol. Endocrinol. Metab.* 285, E1230–E1236.

Grujic, D., Susulic, V. S., Harper, M. E., Himms-Hagen, J., Cunningham, B. A., Corkey, B. E., et al. (1997). β_3-adrenergic receptors on white and brown adipocytes mediate β_3-selective agonist-induced effects on energy expenditure, insulin secretion, and food intake. A study using transgenic and gene knockout mice. *J. Biol. Chem.* 272, 17686–17693.

Hardie, D. G. (2008). AMPK: a key regulator of energy balance in the single cell and the whole organism. *Int. J. Obes. (Lond.)* 32(Suppl. 4), S7–S12.

Hausberg, M., Morgan, D. A., Chapleau, M. A., Sivitz, W. I., Mark, A. L., and Haynes, W. G. (2002). Differential modulation of leptin-induced sympathoexcitation by baroreflex activation. *J. Hypertens.* 20, 1633–1641.

Haynes, W. G., Sivitz, W. I., Morgan, D. A., Walsh, S. A., and Mark, A. L. (1997). Sympathetic and cardiorenal actions of leptin. *Hypertension* 30, 619–623.

Herling, A. W., Kilp, S., Elvert, R., Haschke, G., and Kramer, W. (2008). Increased energy expenditure contributes more to the body weight-reducing effect of rimonabant than reduced food intake in candy-fed wistar rats. *Endocrinology* 149, 2557–2566.

Himms-Hagen, J. (1997). On raising energy expenditure in ob/ob mice. *Science* 276, 1132–1133.

Hoehn, K. L., Turner, N., Swarbrick, M. M., Wilks, D., Preston, E., Phua, Y., et al. (2010). Acute or chronic upregulation of mitochondrial fatty acid oxidation has no net effect on whole-body energy expenditure or adiposity. *Cell Metab.* 11, 70–76.

Holloway, B. R., Howe, R., Rao, B. S., Stribling, D., Mayers, R. M., Briscoe, M. G., et al. (1991). ICI D7114 a novel selective β-adrenoceptor agonist selectively stimulates brown fat and increases whole-body oxygen consumption. *Br. J. Pharmacol.* 104, 97–104.

Hondares, E., Rosell, M., Gonzalez, F. J., Giralt, M., Iglesias, R., and Villarroya, F. (2010). Hepatic FGF21 expression is induced at birth via PPARα in response to milk intake and contributes to thermogenic activation of neonatal brown fat. *Cell Metab.* 11, 206–212.

Horie, L. M., Gonzalez, M. C., Torrinhas, R. S., Cecconello, I., and Waitzberg, D. L. (2011). New specific equation to estimate resting energy expenditure in severely obese patients. *Obesity* 19, 1090–1094.

Inuzuka, M., Tamura, N., Yamada, N., Katsuura, G., Oyamada, N., Taura, D., et al. (2010). C-type natriuretic peptide as a new regulator of food intake and energy expenditure. *Endocrinology* 151, 3633–3642.

James, W. P., Caterson, I. D., Coutinho, W., Finer, N., Van Gaal, L. F., Maggioni, I. P., et al. (2010). Effect of sibutramine on cardiovascular outcomes in overweight and obese subjects. *N. Engl. J. Med.* 363, 905–917.

Jennings, G., Richard, D., and Trayhurn, P. (1986). Effect of caging singly or in groups of different sizes on the thermogenic activity of interscapular brown adipose tissue in mice. *Comp. Biochem. Physiol. A Comp. Physiol.* 85, 583–586.

Kaiyala, K. J., Morton, G. J., Leroux, B. G., Ogimoto, K., Wisse, B., and Schwartz, M. W. (2010). Identification of body fat mass as a major determinant of metabolic rate in mice. *Diabetes* 59, 1657–1666.

Kaiyala, K. J., and Schwartz, M. W. (2011). Toward a more complete (and less controversial) understanding of energy expenditure and its role in obesity pathogenesis. *Diabetes* 60, 17–23.

Kennedy, B. L., and Ellis, S. (1969). Dissociation of catecholamine-induced calorigenesis from lipolysis and glycogenolysis in intact animals. *J. Pharmacol. Exp. Ther.* 168, 137–145.

Klaus, S., Rudolph, B., Dohrmann, C., and Wehr, R. (2005). Expression of uncoupling protein 1 in skeletal muscle decreases muscle energy efficiency and affects thermoregulation and substrate oxidation. *Physiol. Genomics* 21, 193–200.

Klaus, S., Keipert, S., Rossmeisl, M., and Kopecky, J. (2012). Augmenting

energy expenditure by mitochondrial uncoupling: a role of AMP-activated protein kinase. *Genes Nutr.* 7, 369–386.

Klein, J., Fasshauer, M., Benito, M., and Kahn, C. R. (2000). Insulin and the β_3-adrenoceptor differentially regulate uncoupling protein-1 expression. *Mol. Endocrinol.* 14, 764–773.

Koncarevic, A., Kajimura, S., Cornwall-Brady, M., Andreucci, A., Pullen, A., Sako, D., et al. (2012). A novel therapeutic approach to treating obesity through modulation of TGFβ signaling. *Endocrinology* 153, 3133–3146.

Kong, W. M., Martin, N. M., Smith, K. L., Gardiner, J. V., Connoley, I. P., Stephens, D. A., et al. (2004). Triiodothyronine stimulates food intake via the hypothalamic ventromedial nucleus independent of changes in energy expenditure. *Endocrinology* 145, 5252–5258.

Kosari, S., Rathner, J. A., Chen, F., and Badoer, E. (2011). Centrally administered resistin enhances sympathetic nerve activity to the hindlimb but attenuates the activity to brown adipose tissue. *Endocrinology* 152, 2626–2633.

Kunz, I., Meier, M. K., Bourson, A., Fisseha, M., and Schilling, W. (2008). Effects of rimonabant, a cannabinoid CB1 receptor ligand, on energy expenditure in lean rats. *Int. J. Obes. (Lond.)* 32, 863–870.

Kus, V., Prazak, T., Brauner, P., Hensler, M., Kuda, O., Flachs, P., et al. (2008). Induction of muscle thermogenesis by high-fat diet in mice: association with obesity-resistance. *Am. J. Physiol. Endocrinol. Metab.* 295, E356–E367.

Lafrance, L., Routhier, D., Tetu, B., and Tetu, C. (1979). Effects of noradrenaline and nicotinic acid on plasma free fatty acids and oxygen consumption in cold-adapted rats. *Can. J. Physiol. Pharmacol.* 57, 725–730.

Liu, X., Rossmeisl, M., McClaine, J., Riachi, M., Harper, M. E., and Kozak, L. P. (2003). Paradoxical resistance to diet-induced obesity in UCP1-deficient mice. *J. Clin. Invest.* 111, 399–407.

Lou, P. H., Yang, G., Huang, L., Cui, Y., Pourbahrami, T., Radda, G. K., et al. (2010). Reduced body weight and increased energy expenditure in transgenic mice over-expressing soluble leptin receptor. *PLoS ONE* 5:e11669. doi: 10.1371/journal.pone.0011669

Lowell, B. B., Susulic, V. S., Hamann, A., Lawitts, J. A., Himms-Hagen, J., Boyer, B. B., et al. (1993). Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. *Nature* 366, 740–742.

Luheshi, G. N., Gardner, J. D., Rushforth, D. A., Loudon, A. S., and Rothwell, N. J. (1999). Leptin actions on food intake and body temperature are mediated by IL-1. *Proc. Natl. Acad. Sci. U.S.A.* 96, 7047–7052.

Major, G. C., Doucet, E., Trayhurn, P., Astrup, A., and Tremblay, A. (2007). Clinical significance of adaptive thermogenesis. *Int. J. Obes. (Lond.)* 31, 204–212.

Malinowska, B., and Schlicker, E. (1997). Further evidence for differences between cardiac atypical β-adrenoceptors and brown adipose tissue β_3-adrenoceptors in the pithed rat. *Br. J. Pharmacol.* 122, 1307–1314.

Mantzoros, C. S., Qu, D., Frederich, R. C., Susulic, V. S., Lowell, B. B., Maratos-Flier, E., et al. (1996). Activation of β_3-adrenergic receptors suppresses leptin expression and mediates a leptin-independent inhibition of food intake in mice. *Diabetes* 45, 909–914.

Marsh, D. J., Weingarth, D. T., Novi, D. E., Chen, H. Y., Trumbauer, M. E., Chen, A. S., et al. (2002). Melanin-concentrating hormone 1 receptor-deficient mice are lean, hyperactive, and hyperphagic and have altered metabolism. *Proc. Natl. Acad. Sci. U.S.A.* 99, 3240–3245.

Marsicano, G., and Lutz, B. (2006). Neuromodulatory functions of the endocannabinoid system. *J. Endocrinol. Invest.* 29, 27–46.

Mayorov, A. V., Amara, N., Chang, J. Y., Moss, J. A., Hixon, M. S., Ruiz, D. I., et al. (2008). Catalytic antibody degradation of ghrelin increases whole-body metabolic rate and reduces refeeding in fasting mice. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17487–17492.

Mercer, S. W., and Trayhurn, P. (1987). Effect of high fat diets on energy balance and thermogenesis in brown adipose tissue of lean and genetically obese ob/ob mice. *J. Nutr.* 117, 2147–2153.

Meyer, C. W., Willershauser, M., Jastroch, M., Rourke, B. C., Fromme, T., Oelkrug, R., et al. (2010). Adaptive thermogenesis and thermal conductance in wild-type and UCP1-KO mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 299, R1396–R1406.

Mistry, A. M., Swick, A. G., and Romsos, D. R. (1997). Leptin rapidly lowers food intake and elevates metabolic rates in lean and ob/ob mice. *J. Nutr.* 127, 2065–2072.

Mitchell, T. H., Ellis, R. D., Smith, S. A., Robb, G., and Cawthorne, M. A. (1989). Effects of BRL 35135, a β-adrenoceptor agonist with novel selectivity, on glucose tolerance and insulin sensitivity in obese subjects. *Int. J. Obes.* 13, 757–766.

Mnich, S. J., Hiebsch, R. R., Huff, R. M., and Muthian, S. (2010). Anti-inflammatory properties of CB1-receptor antagonist involves β_2 adrenoceptors. *J. Pharmacol. Exp. Ther.* 333, 445–453.

Morrison, S. F. (2001). Differential regulation of sympathetic outflows to vasoconstrictor and thermoregulatory effectors. *Ann. N.Y. Acad. Sci.* 940, 286–298.

Morton, N. M., Paterson, J. M., Masuzaki, H., Holmes, M. C., Staels, B., Fievet, C., et al. (2004). Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11 β-hydroxysteroid dehydrogenase type 1-deficient mice. *Diabetes* 53, 931–938.

Muise, E. S., Azzolina, B., Kuo, D. W., El-Sherbeini, M., Tan, Y., Yuan, X., et al. (2008). Adipose fibroblast growth factor 21 is up-regulated by peroxisome proliferator-activated receptor γ and altered metabolic states. *Mol. Pharmacol.* 74, 403–412.

Murase, T., Misawa, K., Haramizu, S., Minegishi, Y., and Hase, T. (2010). Nootkatone, a characteristic constituent of grapefruit, stimulates energy metabolism and prevents diet-induced obesity by activating AMPK. *Am. J. Physiol. Endocrinol. Metab.* 299, E266–E275.

Myant, N. B., and Witney, S. (1967). The time course of the effect of thyroid hormones upon basal oxygen consumption and plasma concentration of free fatty acid in rats. *J. Physiol.* 190, 221–228.

Ogawa, Y., Masuzaki, H., Hosoda, K., Aizawa-Abe, M., Suga, J., Suda, M., et al. (1999). Increased glucose metabolism and insulin sensitivity in transgenic skinny mice overexpressing leptin. *Diabetes* 48, 1822–1829.

Olson, D. P., Pulinilkunnil, T., Cline, G. W., Shulman, G. I., and Lowell, B. B. (2010). Gene knockout of Acc2 has little effect on body weight, fat mass, or food intake. *Proc. Natl. Acad. Sci. U.S.A.* 107, 7598–7603.

Osaka, T., Tsukamoto, A., Koyama, Y., and Inoue, S. (2008). Central and peripheral administration of amylin induces energy expenditure in anesthetized rats. *Peptides* 29, 1028–1035.

Owen, O. E., Holup, J. L., D'Alessio, D. A., Craig, E. S., Polansky, M., Smalley, K. J., et al. (1987). A reappraisal of the caloric requirements of men. *Am. J. Clin. Nutr.* 46, 875–885.

Picard, F., Deshaies, Y., Lalonde, J., Samson, P., and Richard, D. (2000). Topiramate reduces energy and fat gains in lean (Fa/?) and obese (fa/fa) Zucker rats. *Obes. Res.* 8, 656–663.

Rahmouni, K., and Morgan, D. A. (2007). Hypothalamic arcuate nucleus mediates the sympathetic and arterial pressure responses to leptin. *Hypertension* 49, 647–652.

Ravussin, E., Lillioja, S., Anderson, T. E., Christin, L., and Bogardus, C. (1986). Determinants of 24-hour energy expenditure in man. Methods and results using a respiratory chamber. *J. Clin. Invest.* 78, 1568–1578.

Ravussin, E., Lillioja, S., Knowler, W. C., Christin, L., Freymond, D., Abbott, W. G., et al. (1988). Reduced rate of energy expenditure as a risk factor for body-weight gain. *N. Engl. J. Med.* 318, 467–472.

Reichling, S., Patel, H. V., Freeman, K. B., Kates, A. L., and Himms-Hagen, J. (1988). Attenuated cold-induced increase in mRNA for uncoupling protein in brown adipose tissue of obese (ob/ob) mice. *Biochem. Cell Biol.* 66, 193–198.

Ribeiro, M. O. (2008). Effects of thyroid hormone analogs on lipid metabolism and thermogenesis. *Thyroid* 18, 197–203.

Ribeiro, M. O., Carvalho, S. D., Schultz, J. J., Chiellini, G., Scanlan, T. S., Bianco, A. C., et al. (2001). Thyroid hormone–sympathetic interaction and adaptive thermogenesis are thyroid hormone receptor isoform–specific. *J. Clin. Invest.* 108, 97–105.

Ribeiro, M. O., Bianco, S. D., Kaneshige, M., Schultz, J. J., Cheng, S. Y., Bianco, A. C., et al. (2010). Expression of uncoupling protein 1 in mouse brown adipose tissue is thyroid hormone receptor-β isoform specific and required for adaptive thermogenesis. *Endocrinology* 151, 432–440.

Ricquier, D., Miroux, B., Larose, M., Cassard-Doulcier, A. M., and Bouillaud, F. (2000). Endocrine regulation of uncoupling proteins and energy expenditure. *Int. J. Obes. Relat. Metab. Disord.* 24(Suppl. 2), S86–S88.

Rothwell, N. J., and Stock, M. J. (1987). Influence of clenbuterol on energy balance, thermogenesis and body

composition in lean and genetically obese Zucker rats. *Int. J. Obes.* 11, 641–647.

Rothwell, N. J., and Stock, M. J. (1988). Increased body-weight gain and body protein in castrated and adrenalectomized rats treated with clenbuterol. *Br. J. Nutr.* 60, 355–360.

Rubio, A., Raasmaja, A., Maia, A. L., Kim, K. R., and Silva, J. E. (1995). Effects of thyroid hormone on norepinephrine signaling in brown adipose tissue. I. β_1- and β_2-adrenergic receptors and cyclic adenosine 3', 5'-monophosphate generation. *Endocrinology* 136, 3267–3276.

Rucker, D., Padwal, R., Li, S. K., Curioni, C., and Lau, D. C. (2007). Long term pharmacotherapy for obesity and overweight: updated meta-analysis. *BMJ* 335, 1194–1199.

Russell, S. T., Hirai, K., and Tisdale, M. J. (2002). Role of β_3-adrenergic receptors in the action of a tumour lipid mobilizing factor. *Br. J. Cancer* 86, 424–428.

Russell, S. T., and Tisdale, M. J. (2011a). Studies on the anti-obesity activity of zinc-α_2-glycoprotein in the rat. *Int. J. Obes. (Lond.)* 35, 658–665.

Russell, S. T., and Tisdale, M. J. (2011b). Studies on the antiobesity effect of zinc-α_2-glycoprotein in the ob/ob mouse. *Int. J. Obes. (Lond.)* 35, 345–354.

Russell, S. T., and Tisdale, M. J. (2012a). Role of β-adrenergic receptors in the anti-obesity and anti-diabetic effects of zinc-α_2-glycoprotien (ZAG). *Biochim. Biophys. Acta* 1821, 590–599.

Russell, S. T., and Tisdale, M. J. (2012b). Role of β-adrenergic receptors in the oral activity of Zinc-α_2-Glycoprotein (ZAG). *Endocrinology* 153, 4696–4704.

Schiffelers, S. L., Blaak, E. E., Saris, W. H., and van Baak, M. A. (2000). *In vivo* β_3-adrenergic stimulation of human thermogenesis and lipid use. *Clin. Pharmacol. Ther.* 67, 558–566.

Schiffelers, S. L., Brouwer, E. M., Saris, W. H., and van Baak, M. A. (1998). Inhibition of lipolysis reduces β_1-adrenoceptor-mediated thermogenesis in man. *Metab. Clin. Exp.* 47, 1462–1467.

Schiffelers, S. L., Saris, W. H., Boomsma, F., and van Baak, M. A. (2001). β_1- and β_2-Adrenoceptor-mediated thermogenesis and lipid utilization in obese and lean men. *J. Clin. Endocrinol. Metab.* 86, 2191–2199.

Schuhler, S., Warner, A., Finney, N., Bennett, G. W., Ebling, F. J., and Brameld, J. M. (2007). Thyrotrophin-releasing hormone decreases feeding and increases body temperature, activity and oxygen consumption in Siberian hamsters. *J. Neuroendocrinol.* 19, 239–249.

Selman, C., Lumsden, S., Bunger, L., Hill, W. G., and Speakman, J. R. (2001). Resting metabolic rate and morphology in mice (*Mus musculus*) selected for high and low food intake. *J. Exp. Biol.* 204, 777–784.

Shintani, M., Tamura, Y., Monden, M., and Shiomi, H. (2005). Thyrotropin-releasing hormone induced thermogenesis in Syrian hamsters: site of action and receptor subtype. *Brain Res.* 1039, 22–29.

Silva, J. E. (2006). Thermogenic mechanisms and their hormonal regulation. *Physiol. Rev.* 86, 435–464.

Smith, S. A., Sennitt, M. V., and Cawthorne, M. A. (1990). "BRL 35135: an orally active antihyperglycaemic agent with weight reducing effects," in *New Antidiabetic Drugs*, eds C. J. Bailey and P. R. Flatt (London: Smith-Gordon), 177–189.

Speakman, J. R. (2013). Measuring energy metabolism in the mouse – theoretical, practical and analytical considerations. *Front. Physiol.* 4:34. doi: 10.3389/fphys.2013.00034

Speakman, J. R., and Johnson, M. S. (2000). "Relationships between resting metabolic rate and morphology in lactating mice: what tissues are the major contributors to resting metabolism?" in *Life in the Cold*, eds G. Heldmaier and M. Klingenspor (Berlin: Springer), 479–486.

Stehling, O., Doring, H., Ertl, J., Preibisch, G., and Schmidt, I. (1996). Leptin reduces juvenile fat stores by altering the circadian cycle of energy expenditure. *Am. J. Physiol.* 271, R1770–R1774.

Strubelt, O., and Siegers, C. P. (1969). [On the mechanism of the calorigenic effect of theophylline and caffeine]. *Biochem. Pharmacol.* 18, 1207–1220.

Takahashi, N., Li, F., Hua, K., Deng, J., Wang, C. H., Bowers, R. R., et al. (2007). Increased energy expenditure, dietary fat wasting, and resistance to diet-induced obesity in mice lacking renin. *Cell Metab.* 6, 506–512.

Tam, J., Cinar, R., Liu, J., Godlewski, G., Wesley, D., Jourdan, T., et al. (2012). Peripheral cannabinoid-1 receptor inverse agonism reduces obesity by reversing leptin resistance. *Cell Metab.* 16, 167–179.

Thurlby, P. L., and Ellis, R. D. (1986). Differences between the effects of noradrenaline and the β-adrenoceptor agonist BRL 28410 in brown adipose tissue and hind limb of the anaesthetized rat. *Can. J. Physiol. Pharmacol.* 64, 1111–1114.

Thurlby, P. L., and Trayhurn, P. (1979). The role of thermoregulatory thermogenesis in the development of obesity in genetically-obese (ob/ob) mice pair-fed with lean siblings. *Br. J. Nutr.* 42, 377–385.

Tomlinson, E., Fu, L., John, L., Hultgren, B., Huang, X., Renz, M., et al. (2002). Transgenic mice expressing human fibroblast growth factor-19 display increased metabolic rate and decreased adiposity. *Endocrinology* 143, 1741–1747.

Trayhurn, P., and James, W. P. (1978). Thermoregulation and non-shivering thermogenesis in the genetically obese (ob/ob) mouse. *Pflugers Arch.* 373, 189–193.

Trayhurn, P., and Milner, R. E. (1989). A commentary on the interpretation of *in vitro* biochemical measures of brown adipose tissue thermogenesis. *Can. J. Physiol. Pharmacol.* 67, 811–819.

Trayhurn, P., Duncan, J. S., Rayner, D. V., and Hardie, L. J. (1996). Rapid inhibition of ob gene expression and circulating leptin levels in lean mice by the β_3-adrenoceptor agonists BRL 35135A and ZD2079. *Biochem. Biophys. Res. Commun.* 228, 605–610.

Tschop, M. H., Speakman, J. R., Arch, J. R., Auwerx, J., Bruning, J. C., Chan, L., et al. (2012). A guide to analysis of mouse energy metabolism. *Nat. Methods* 9, 57–63.

Ueta, C. B., Fernandes, G. W., Capelo, L. P., Fonseca, T. L., Maculan, F. D., Gouveia, C. H., et al. (2012). β_1 Adrenergic receptor is key to cold- and diet-induced thermogenesis in mice. *J. Endocrinol.* 214, 359–365.

Ukropec, J., Anunciado, R. P., Ravussin, Y., Hulver, M. W., and Kozak, L. P. (2006a). UCP1-independent thermogenesis in white adipose tissue of cold-acclimated Ucp1-/- mice. *J. Biol. Chem.* 281, 31894–31908.

Ukropec, J., Anunciado, R. V., Ravussin, Y., and Kozak, L. P. (2006b). Leptin is required for uncoupling protein-1-independent thermogenesis during cold stress. *Endocrinology* 147, 2468–2480.

Verty, A. N., Allen, A. M., and Oldfield, B. J. (2009). The effects of rimonabant on brown adipose tissue in rat: implications for energy expenditure. *Obesity* 17, 254–261.

Vosselman, M. J., van der Lans, A. A., Brans, B., Wierts, R., van Baak, M. A., Schrauwen, P., et al. (2012). Systemic β-adrenergic stimulation of thermogenesis is not accompanied by brown adipose tissue activity in humans. *Diabetes* 61, 3106–3113.

Wang, S. J., Birtles, S., de Schoolmeester, J., Swales, J., Moody, G., Hislop, D., et al. (2006). Inhibition of 11β-hydroxysteroid dehydrogenase type 1 reduces food intake and weight gain but maintains energy expenditure in diet-induced obese mice. *Diabetologia* 49, 1333–1337.

Wang, S. J., Cornick, C., O'Dowd, J., Cawthorne, M. A., and Arch, J. R. (2007). Improved glucose tolerance in acyl CoA:diacylglycerol acyltransferase 1-null mice is dependent on diet. *Lipids Health Dis.* 6:2. doi: 10.1186/1476-511X-6-2

Wang, Z., Heymsfield, S. B., Ying, Z., Pierson, R. N. Jr., Gallagher, D., and Gidwani, S. (2010a). A cellular level approach to predicting resting energy expenditure: Evaluation of applicability in adolescents. *Am. J. Hum. Biol.* 22, 476–483.

Wang, Z., Ying, Z., Bosy-Westphal, A., Zhang, J., Schautz, B., Later, W., et al. (2010b). Specific metabolic rates of major organs and tissues across adulthood: evaluation by mechanistic model of resting energy expenditure. *Am. J. Clin. Nutr.* 92, 1369–1377.

Wargent, E. T., O'Dowd, J. F., Zaibi, M. S., Gao, D., Bing, C., Trayhurn, P., et al. (2013). Contrasts between the effects of zinc-α_2-glycoprotein, a putative β_3/2-adrenoceptor agonist and the β_3/2-adrenoceptor agonist BRL35135 in C57Bl/6 (ob/ob) mice. *J. Endocrinol.* 216, 157–168.

Watanabe, M., Houten, S. M., Mataki, C., Christoffolete, M. A., Kim, B. W., Sato, H., et al. (2006). Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature* 439, 484–489.

Wellman, P. J., and Marmon, M. M. (1985). Synergism between caffeine and dl-phenylpropanolamine on brown adipose tissue thermogenesis in the adult rat. *Pharmacol. Biochem. Behav.* 22, 781–785.

Wellman, P. J., Marmon, M. M., Reich, S., and Ruddle, J. (1986). Effects of nicotine on body weight, food intake and brown adipose tissue thermogenesis. *Pharmacol. Biochem. Behav.* 24, 1605–1609.

Wernstedt, I., Edgley, A., Berndtsson, A., Faldt, J., Bergstrom, G., Wallenius, V., et al. (2006). Reduced stress- and cold-induced increase in

energy expenditure in interleukin-6-deficient mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 291, R551–R557.

Whittle, A. J., Carobbio, S., Martins, L., Slawik, M., Hondares, E., Vazquez, M. J., et al. (2012). BMP8B increases brown adipose tissue thermogenesis through both central and peripheral actions. *Cell* 149, 871–885.

Wilson, S., Arch, J. R., and Thurlby, P. L. (1984). Genetically obese C57BL/6 ob/ob mice respond normally to sympathomimetic compounds. *Life Sci.* 35, 1301–1309.

Wong, D., Sullivan, K., and Heap, G. (2012). The pharmaceutical market for obesity therapies. *Nat. Rev. Drug Discov.* 11, 669–670.

Wu, A. L., Kolumam, G., Stawicki, S., Chen, Y., Li, J., Zavala-Solorio, J., et al. (2011). Amelioration of type 2 diabetes by antibody-mediated activation of fibroblast growth factor receptor 1. *Sci. Transl. Med.* 3, 113ra126.

Xu, J., Lloyd, D. J., Hale, C., Stanislaus, S., Chen, M., Sivits, G., et al. (2009). Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice. *Diabetes* 58, 250–259.

Ye, L., Kleiner, S., Wu, J., Sah, R., Gupta, R. K., Banks, A. S., et al. (2012). TRPV4 Is a Regulator of adipose oxidative metabolism, inflammation, and energy homeostasis. *Cell* 151, 96–110.

Yoshida, T., Umekawa, T., Kumamoto, K., Sakane, N., Kogure, A., Kondo, M., et al. (1998). β_3-Adrenergic agonist induces a functionally active uncoupling protein in fat and slow-twitch muscle fibers. *Am. J. Physiol.* 274, E469–E475.

Yoshioka, K., Yoshida, T., Kamanaru, K., Hiraoka, N., and Kondo, M. (1990). Caffeine activates brown adipose tissue thermogenesis and metabolic rate in mice. *J. Nutr. Sci. Vitaminol.* 36, 173–178.

Zhang, J., Zhao, J., Jiang, W. J., Shan, X. W., Yang, X. M., and Gao, J. G. (2012a). Conditional gene manipulation: cre-ating a new biological era. *J. Zhejiang Univ. Sci. B* 13, 511–524.

Zhang, Y., Xie, Y., Berglund, E. D., Coate, K. C., He, T. T., Katafuchi, T., et al. (2012b). The starvation hormone, fibroblast growth factor-21, extends lifespan in mice. *Elife* 1:e00065. doi: 10.7554/eLife.00065

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 January 2013; paper pending published: 07 February 2013; accepted: 16 March 2013; published online: 08 April 2013.

*Citation: Arch JRS and Trayhurn P (2013) Detection of thermogenesis in rodents in response to anti-obesity drugs and genetic modification. Front. Physiol. **4**:64. doi: 10.3389/fphys.2013.00064*

This article was submitted to Frontiers in Integrative Physiology, a specialty of Frontiers in Physiology.

frontiers in
PHYSIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 16 December 2011
doi: 10.3389/fphys.2011.00096

Identification of behavioral and metabolic factors predicting adiposity sensitivity to both high fat and high carbohydrate diets in rats

Patrick Christian Even*, Nachiket A. Nadkarni, Catherine Chaumontet, Dalila Azzout-Marniche, Gilles Fromentin and Daniel Tomé

UMR914 Nutrition Physiology and Ingestive Behavior, AgroParisTech, INRA, Paris, France

Edited by:
Jean-Pierre Montani, University of Fribourg, Switzerland

Reviewed by:
Jean-Pierre Montani, University of Fribourg, Switzerland
Catalina Pico, University of the Balearic Islands, Spain
Gretha Boersma, Johns Hopkins University, USA

****Correspondence:***
Patrick Christian Even, UMR914 Nutrition Physiology and Ingestive Behavior, AgroParisTech, INRA, F-75005 Paris, France.
e-mail: even@agroparistech.fr

Individuals exhibit a great variation in their body weight (BW) gain response to a high fat diet. Identification of predictive factors would enable better directed intervention toward susceptible individuals to treat obesity, and uncover potential mechanisms for treatment targeting. We set out to identify predictive behavioral and metabolic factors in an outbred rat model. 12 rats were analyzed in metabolic cages for a period of 5 days during both high carbohydrate diet (HCD), and transition to a high fat diet (HFD). After a recovery period, rats were given a HFD for 6 days to identify those resistant or sensitive to it according to BW gain. Rats were dissected at the end of the study to analyze body composition. This showed that small differences in final BW hid large variations in adiposity, allowing separation of rats into a second classification (final adiposity). Since these rats had been fed a HCD during most of their life, under which most of the adiposity presumably evolved, we considered this carbohydrate-sensitivity or -resistance. Meal size and meal number were found to be good predictors of sensitivity to a HFD, intensity of motor activity and ingestion speed good predictors of sensitivity to a HCD. Rats that were sensitive to the HCD could be resistant to the HFD and vice versa. This points to four types of individuals (carbohydrate/fat resistant/sensitive) though our sample size inhibited deeper investigation of this. This contributes to the idea that to be "obesity prone" does not necessarily need a HFD, it can also happen under a HCD, and be a hidden adiposity change with stable BW.

Keywords: obesity prone, obesity resistant, rat, food intake, motor activity, energy metabolism, high fat diet/low fat diet, indirect calorimetry

INTRODUCTION

Stability of body weight (BW) and body composition requires that over time, energy expenditure equals caloric intake (CI), in other words that energy nutrient oxidation equals intake in order to achieve nutrient balance. There is however a great inter-individual variability in human as well as in many animal populations in the capacity to achieve this (Chang et al., 1990; Neel, 1999; Speakman, 2007; Prentice et al., 2008; Stoger, 2008).

Because of extensive metabolic capacities to store fat in adipose tissue, fat balance is usually difficult to achieve more particularly in some fat-sensitive (FS; FR being resistant) subjects for whom high fat diets (HFD) promote obesity (Flatt, 1987, 1988). Moreover, as glucose is the main precursor for lipogenesis, some sensitive subjects could efficiently convert glucose to fat (subsequently stored in adipose tissue) thus also leading to difficulty to achieve energy balance, whereas other less sensitive subjects could more readily adjust carbohydrate oxidation to carbohydrate intake. Accordingly, high carbohydrate diets (HCD) could also promote obesity in carbohydrate-sensitive (CS; CR being resistant) subjects. In contrast, despite the fact that the underlying mechanisms are not clarified, it is clearly established that high protein diets (HPD) do not promote obesity (Westerterp-Plantenga et al., 2001; Lacroix et al., 2004; Pichon et al., 2006; Layman et al., 2009).

The mechanisms potentially responsible for the sensitivity to fat have been extensively investigated using genetically bred FS rats that offer a well-defined population in the study of metabolic and behavioral characteristics related to sustained sensitivity or resistance to fat (Levin et al., 1997). Another approach is also to select subjects in a population of outbred rats (Chang et al., 1990). Selection is based on the level of sensitivity to HFD and additionally metabolic and/or behavioral component(s) that participate in this sensitivity. Many differences have been reported in separate studies in FS rats such as abnormal sympathetic nervous activity, increased release of norepinephrine after a glucose load, impaired growth hormone secretion, and suppression of glucose appearance by insulin (Ji and Friedman, 2003). It is not clear whether all these differences are present simultaneously in all FS rats or more probably reflect various defects acting alone or in conjunction to favor body fat gain, thus suggesting a complex distribution of rats according to their sensitivity to body fat accretion.

Abbreviations: BW, body weight; CI, caloric intake; CR, carbohydrate resistant; CS, carbohydrate sensitive; EE, energy expenditure; FI, food intake; FR, fat resistant; FS, fat sensitive; HCD, high carbohydrate diet; HFD, high fat diet; HPD, high protein diet; IMI, inter meal interval; MS, meal size; RQ, respiratory quotient; SPA, spontaneous physical activity; TEE, total energy expenditure.

This study hypothesized that differences can be observed not only under HFD (FS vs. FR) but also under HCD (CS vs. CS). Under these conditions, this work aims to characterize rats according to their relative resistance to fat gain under HFD or HCD and to investigate if various metabolic and/or behavioral parameters easily accessible before the rats are overweight and when fed a standard HCD can be good predictors of the specific sensitivity of individuals to HFD or HCD. For this purpose energy expenditure and behavioral differences were determined between FS and FR rats fed a low fat diet (HCD) and submitted to a short period of HFD. The experiment aimed to measure total energy expenditure (TEE), CI, and spontaneous physical activity (SPA) under free-feeding conditions during HCD and transition to HFD.

EXPERIMENTAL METHODS

ANIMALS, HOUSING, AND DIETS

The experimental protocol was approved by the French National Animal Care Committee. Male Wistar rats (Harlan Laboratories) weighed around 225 g (7 weeks) at their arrival in the laboratory. With the goal of keeping all the rats in the study and thus to avoid sacrificing many animals, the breeder was asked to select from its colony of 7-week-old rats the six lightest and the six heaviest in order to get rats with a large phenotypic heterogeneity. The composition of the diets is presented in **Table 1**. Animal facility conditions were a 12:12 Light-Dark cycle and 22°C.

DESIGN

This is outlined in **Figure 1**. The rats were weighed 2 days after arrival in the laboratory. After the selection performed by the breeder, the range of BWs of the rats was large (206–258 g) but the mean weights of the light and heavy rats not substantially different (219.8 ± 5.1 vs 233.5 ± 4.6, $P = 0.07$) with individuals overlapping between the two groups. After arrival and 5 days of adaptation to the animal facility, the 12 rats were split into three groups of 4 rats (the number of rats that could be studied simultaneously in the behavioral-metabolic device) and maintained under HCD in order to be studied in turn by group (1, 2, and 3 weeks after arrival in the laboratory). During a 5-day period each of the three groups was characterized for TEE, food intake (FI), and SPA during transition from HCD to HFD. After completion of the 5-days of calorimetric measurements, the rats were allowed to recover for 10 days under HCD, and then fed the HFD for 6 days to allow selection from BW gain between FS and FR rats. Body composition was analyzed from dissection and weighing of the main organs and tissues just after the end of the 6-day HFD period. These 12 rats could readily be separated into FS and FR groups, each of six rats with a highly significant difference in their BW gain response to the HFD. This suggested that despite initial small differences in BW, the selection performed by the breeder helped in the creation of a group with a large heterogeneity. There was however no correlation between the initial weight of the rats and their weight gain during the HFD period.

Table 1 | Nutrient composition of the high carbohydrate (HCD) and high fat (HFD) diets.

	HCD	HFD
	Amount (g)	
Milk protein	140.0	170.0
Corn starch	622.4	436.6
Saccharose	100.3	71.1
Soybean oil	40.0	225.0
Mineral mix	35.0	35.0
Vitamin mix	10.0	10.0
Cellulose	50.0	50.0
Choline	2.3	2.3
Total	1000	1000
	Energy (%)	
Milk protein	14.7	14.4
Corn starch	65.3	36.9
Saccharose	10.5	6.0
Soybean oil	9.4	42.8
Total	100	100
Energy (kJ/g)	15.95	19.82
Food quotient	0.946	0.847

MEASUREMENT OF THE COMPONENTS OF ENERGY EXPENDITURE

The goal was to obtain for each rat measures of FI pattern, SPA, TEE and respiratory quotient (RQ) during HCD, HFD and the transition between the two. Groups of four rats were housed at 18:00 in individual metabolic cages equipped with a weighed food cup (sensitivity better than 0.05 g) and an activity platform placed below (sensitivity better than 1 g). For gas analysis, the cages were multiplexed – all connected to the same gas analyzers. Thus VO_2 and VCO_2 were measured on each cage during 2 min every 10 min (2 min for each cage, plus 2 min on room air to correct values for room $\%O_2$ and $\%CO_2$). To reduce close to zero any energy expenditure for thermoregulation (non-shivering thermogenesis), temperature in the room was maintained at 25–26°C in order to maintain in the metabolic cage a temperature of 26–27°C.

Day 1 in the metabolic device was used for habituation. VO_2–VCO_2 and FI were measured during day 2 (HCD feeding) and during days 3–5 the rats were switched to the HFD. For each cage FI and SPA were measured in 5 s time bins on a separate computer. For analysis, data were pooled into 10 min bins and combined with the VO_2–VCO_2 data. Metabolic rate was computed from VO_2 and VCO_2 according to the Weir formula (Ferrannini, 1988; Even et al., 1994).

ANALYSIS OF BODY COMPOSITION

Rats were deeply anesthetized with an overdose of pentobarbital (40 mg/kg) then killed following exsanguination by blood sampling from the vena cava. The main tissues and organs (liver, spleen, kidneys, brain, heart, subcutaneous, retroperitoneal, inguinal, and mesenteric fat pads, skin) were dissected out, blotted dry, and weighed to the nearest 0.01 g.

STATISTICAL ANALYSIS

Differences between groups (FR vs. FS and CR vs. CS) were assessed using Student's t-test. A difference was considered statistically significant at $P < 0.025$ (Bonferroni correction) to take

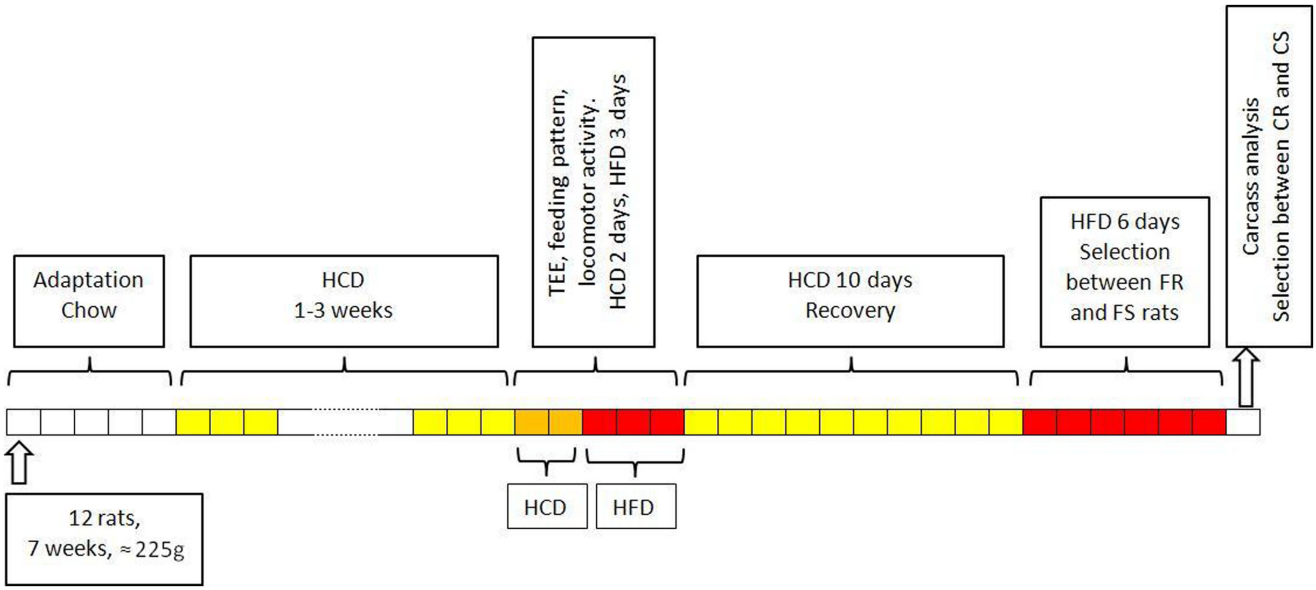


FIGURE 1 | Study design.

Table 2 | Body weight, composition, and weight gain of the FR and FS or CR and CS rats during the various periods (numbers of rats in each group in parentheses).

	FR vs. FS					CR vs. CS				
	FR(6)		FS(6)		*P*	CR(5)		CS(7)		*P*
	Mean	SE	Mean	SE		Mean	SE	Mean	SE	
Body weight gain during HFD (6 days)	21.67	2.02	31.15	1.80	0.006	27.42	4.10	25.69	1.86	NS
Final BW (g)	353.85	8.58	354.70	20.60	NS	336.34	8.40	367.09	16.02	NS
LBM (g)	307.29	4.41	306.20	15.96	NS	300.22	6.05	311.41	13.03	NS
Carcass mass (g)	152.39	3.40	153.15	9.46	NS	148.84	3.94	155.57	7.85	NS
Body fat (g)	46.56	4.90	48.51	6.47	NS	36.12	4.19	55.68	3.52	0.006
Adiposity (%)	13.04	1.12	13.50	1.32	NS	10.68	1.07	15.12	0.49	0.010

SE, standard error (of the mean). P, result of a two-tailed Student's t-test, d.f. = 10. NS, not significant.

into account that the tests were repeated in the FR–FS and CR–CS study. Pearson product moment correlation analysis was also used to examine the relationship between the level of sensitivity to fat or carbohydrates and various metabolic and behavioral parameters that showed a trend toward significance between FR/FS or CR/CS rats.

RESULTS

CLASSIFICATION OF FS VS. FR AND CS VS. CR RATS ACCORDING TO BW GAIN AND BODY COMPOSITION

Body weight and composition data are shown in **Table 2**. The six FS rats were selected according to their larger daily BW gain during the period of HFD. Accordingly, BW gain during HF feeding was 44% larger in the FS than in the FR rats but no differences in body adiposity were observed between the two groups at the end of the study. However, within each group and therefore amongst all 12 rats combined, there was a great variation in adiposity. This allowed a second classification according to adiposity into five leaner rats (mean adiposity $10.68 \pm 1.07\%$) and seven fattier rats (mean adiposity $15.12 \pm 0.49\%$). We refer to the leaner rats as CR and to the fattier as CS since the adiposity differences were accumulated in rats fed under HCD during most of their life. In addition, because the CR and CS rats were approximately evenly distributed (five vs seven) into the FS and FR groups, we assumed that the 6-days of HFD affected similarly the body fat gain during this period in the CR and CS rats. Despite these large differences in body fat and body adiposity, final BWs of the CS and CR rats were not significantly different. Of the 31-g difference in BW, 20 g was accounted for by body fat (+53% in the CS rats) while only 10 g was accounted for by LBM (+3.7% in the CS rats). BW gain during HF feeding was also very similar in CR and CS rats.

COMPARISON OF THE COMPONENTS OF ENERGY EXPENDITURE FOR RATS CLASSIFIED AS FS VS. FR

The meal pattern under HCD in rats classified as FS was characterized by significantly more numerous meals compared with FR during HCD but the difference decreased during HFD (**Table 3**). In parallel, inter-meal interval (IMI) and meal size tended to be lower which explains the absence of a significantly higher overall CI. No differences were observed between FR and FS rats for the components of SPA (**Table 4**). TEE was similar in FS and FR rats under HCD, but decreased progressively in FS rats during HFD and became significantly lower than in FR rats after 3 days (**Table 5**). When expressed relative to HCD, TEE during HFD was significantly more decreased in FS rats after 2 and 3 days of HFD. In parallel, RQ tended to decrease more in FR rats, indicating a better adaptation to the increased fat content of the diet, and became significantly lower in FR than FS rats after 3 days of HFD. In summary, FS rats can be distinguished from FR rats from their larger meal number under HCD, and a larger decrease in TEE and maintenance of a higher RQ in response to HFD.

COMPARISON OF THE COMPONENTS OF ENERGY EXPENDITURE FOR RATS CLASSIFIED AS CS VS. CR

None of the parameters of FI showed significant differences between CR and CS rats during HCD as well as during HFD. In both groups, HFD increased CI. CS rats tended to spend more time active than CR rats during HCD (+23%, $P = 0.054$; **Table 4**), but this tendency disappeared completely during HFD. In contrast, during HCD CS rats developed bursts of SPA that were of significantly lower intensity (−29%, $P = 0.004$,) than those recorded in the CR rats. This characteristic vanished progressively under HFD. No differences were observed between the two groups in TEE or RQ throughout the 4-days (**Table 5**). In summary, CS rats can be characterized primarily from the lower intensity of their bursts of SPA relative to CR rats during HCD.

PREDICTION POTENTIAL OF THE METABOLIC AND BEHAVIORAL PARAMETERS FOR FS/FR AND CS/CR RATS

According to the differences observed in the FS/FR and CS/CR rats, we re-analyzed the data starting from the potential predictive parameters to test if these parameters could indeed be good predictive factors.

Predicting the FS/FR phenotype

Fat-sensitive rats appeared to eat significantly more meals when fed the HCD than FR rats. Classification of the rats in accordance with meal number required that one rat that ate a large number of meals ($n = 21$) and was initially in the FR group had to be included in the group of potentially FS rats. This rat was lean (body fat 8.92%), but was indeed the FR rat that exhibited the largest BW gain during the 6-days of HF feeding (25.5 g). Recalculation of BW gain and body composition of the two groups thus created according to meal frequency showed that they were indeed characterized by a very significant difference in weight gain during HF feeding (FS vs. FR; 29.00 ± 1.28 vs. 22.43 ± 2.21 g, $P = 0.024$, d.f. $= 10$).

Table 3 | Evolution of meal patterns in FS, FR and CR, CS rats in Design 2.

		FR vs. FS					CR vs. CS				
		FR(6)		FS(6)		*P*	CR(5)		CS(7)		*P*
		Mean	SE	Mean	SE		Mean	SE	Mean	SE	
Caloric intake (kJ)	HCD	293.30	17.99	312.54	6.69	NS	293.72	22.59	309.20	5.86	NS
	HFD day 1	374.05	13.39	360.66	7.95	NS	361.50	10.46	371.54	11.72	NS
	HFD day 2	389.53	15.90	359.41	15.48	NS	360.66	23.01	384.09	11.30	NS
	HFD day 3	263.17	39.75	292.46	43.51	NS	221.75	10.04	317.57	43.10	NS
Meal number	HCD	15.3	1.6	21.3	1.1	0.013	19.0	1.6	17.9	2.0	NS
	HFD day 1	15.2	1.2	20.8	1.7	0.023	17.8	1.6	18.1	2.0	NS
	HFD day 2	13.0	1.0	18.2	1.7	NS	15.0	1.3	16.0	1.9	NS
	HFD day 3	9.8	0.9	13.2	1.2	NS	10.8	1.0	12.0	1.3	NS
IMI (min)	HCD	72.6	6.5	54.8	4.4	NS	58.5	5.2	67.4	6.9	NS
	HFD day 1	84.1	5.9	61.1	5.0	0.015	72.3	6.5	72.6	7.8	NS
	HFD day 2	87.2	8.1	73.4	6.9	NS	77.5	6.4	82.3	8.5	NS
	HFD day 3	103.9	6.9	92.1	6.4	NS	97.4	4.5	98.5	7.9	NS
Meal size (kJ)	HCD	20.75	3.18	14.85	0.96	NS	16.19	2.47	19.00	2.64	NS
	HFD day 1	25.36	1.84	17.99	1.72	0.015	21.05	2.01	22.13	2.55	NS
	HFD day 2	30.84	2.68	21.00	2.80	NS	24.89	2.80	26.65	3.68	NS
	HFD day 3	26.78	3.01	24.02	5.48	NS	21.05	1.38	28.49	4.85	NS
Speed (kJ/min)	HCD	2.50	0.27	2.49	0.21	NS	2.18	0.26	2.72	0.18	NS
	HFD day 1	3.90	0.30	5.40	1.03	NS	4.07	0.46	5.06	0.90	NS
	HFD day 2	4.23	0.63	4.48	0.21	NS	3.98	0.51	4.64	0.41	NS
	HFD day 3	4.10	0.88	3.89	0.59	NS	3.53	0.48	4.31	0.81	NS

SE, standard error (of the mean). P, result of a two-tailed Student's t-test, d.f. = 10. IMI, inter-meal interval. NS, not significant.

Table 4 | Components of spontaneous activity in FS, FR and CR, CS rats in Design 2 (numbers of rats in each group in parentheses).

	Whole day activity	FR vs. FS					CR vs. CS				
		FR(6)		FS(6)		*P*	CR (5)		CS (7)		*P*
		Mean	SE	Mean	SE		Mean	SE	Mean	SE	
Time active (min)	HCD	62.19	5.97	60.65	4.32	NS	54.119	1.655	66.635	5.176	0.054
	HFD day 1	64.57	4.43	75.92	4.18	NS	69.284	5.779	70.938	4.427	NS
	HFD day 2	63.11	4.67	74.85	4.65	NS	66.887	5.608	70.476	4.997	NS
	HFD day 3	65.41	6.45	57.13	1.37	NS	65.296	5.486	58.395	4.280	NS
Mean act (U/100 g)	HCD	1.777	0.140	1.904	0.161	NS	2.024	0.147	1.710	0.129	NS
	HFD day 1	1.828	0.129	1.886	0.178	NS	2.089	0.157	1.692	0.109	NS
	HFD day 2	1.808	0.092	2.041	0.147	NS	2.119	0.098	1.785	0.115	NS
	HFD day 3	2.118	0.156	2.012	0.152	NS	2.301	0.054	1.897	0.148	NS
Intensity of bursts (U/100 g)	HCD	2.962	0.326	3.206	0.328	NS	3.723	0.167	2.627	0.246	0.004
	HFD day 1	2.888	0.266	2.481	0.163	NS	3.080	0.282	2.402	0.108	NS
	HFD day 2	2.918	0.210	2.832	0.364	NS	3.241	0.254	2.613	0.262	NS
	HFD day 3	3.302	0.214	3.540	0.307	NS	3.600	0.235	3.293	0.267	NS

Activity is measured by means of an activity platform in which force transducers record the work developed on the floor of the cage while the animal is active (see Even et al., 1994). Statistical analysis of the distribution of the activity signal gives a Poisson-like shaped curve from which can be statistically computed the threshold intensity between "resting" and "activity" values. "Mean act" is the average intensity of activity and includes the periods of inactivity. It is proportional to the daily work expended with activity. "Intensity of bursts" is the average intensity of activity developed during the periods of activity. SE, standard error (of the mean). P, result of a two-tailed Student's t-test, d.f. = 10.

Table 5 | Components of energy expenditure in FR and FS or CR and CS rats during low and high fat feeding in Design 2 (numbers of rats in each group in parentheses).

		FR vs. FS					CR vs. CS				
		FR(6)		FS(6)		*P*	CR(5)		CS(7)		*P*
		Mean	SE	Mean	SE		Mean	SE	Mean	SE	
EE (kJ)	HCD	232.30	2.76	242.96	6.74	NS	236.77	5.27	238.28	5.69	NS
	HFD day 1	219.95	5.27	230.87	5.73	NS	222.84	7.03	227.23	5.19	NS
	HFD day 2	230.62	3.31	228.45	6.65	NS	231.33	3.22	228.24	5.86	NS
	HFD day 3	230.54	2.59	219.79	8.79	0.01	227.07	5.69	223.80	7.20	NS
dEE vs. HCD (%)	HFD day 1	−22.51	5.31	−20.63	3.18	NS	−24.77	6.53	−19.25	2.26	NS
	HFD day 2	−2.93	5.82	−24.98	2.97	0.01	−8.87	9.41	−17.57	4.23	NS
	HFD day 3	−2.89	7.03	−40.33	6.40	0.00	−15.98	15.69	−25.61	6.40	NS
RQ	HCD	1.028	0.016	1.033	0.007	NS	1.018	0.019	1.040	0.006	NS
	HFD day 1	0.955	0.008	0.982	0.013	NS	0.965	0.015	0.971	0.010	NS
	HFD day 2	0.943	0.005	0.955	0.011	NS	0.946	0.011	0.951	0.008	NS
	HFD day 3	0.91	0.01	0.95	0.00	0.01	0.92	0.01	0.94	0.01	NS
dRQ vs. HCD (%)	HFD day 1	−7.08	0.85	−4.96	0.87	NS	−5.15	0.91	−6.64	0.91	NS
	HFD day 2	−8.25	1.21	−7.56	0.54	NS	−6.96	1.03	−8.58	0.78	NS
	HFD day 3	−11.29	1.87	−8.04	0.44	NS	−9.35	1.80	−9.88	1.35	NS

For a better adjustment of energy expenditure between rats, we avoided adjustment of energy expenditure based on whole body weight, and instead used LBM. This is because carcass analysis performed only 2 weeks after the calorimetric studies revealed that 2/3 of the differences in body weight were accounted for by differences in body fat. We thus assumed that body adiposity during the calorimetric studies was not very different from that measured from carcass analysis. The computed LBM value was used to normalize energy expenditure (EE) between rats. SE, standard error (of the mean). P, result of a two-tailed Student's t-test, d.f. = 10.

Analysis of their metabolic and behavioral characteristics logically increased the differences under HCD (but also under HFD) in meal number (21.5 ± 1.1 vs. 13.5 ± 1.6, $P = 0.002$, d.f. $= 10$ during HCD), MS (13.9 ± 1.17 vs. 24.5 ± 3.18 kJ, $P = 0.007$, d.f. $= 10$), and IMI (53.5 ± 4.3 vs. 81.0 ± 5.6 min, $P = 0.004$, d.f. $= 10$), which became significantly different. The decrease in RQ measured after

3 days of HF feeding was still smaller in FS rats (-7.374 ± 0.552 vs. -13.042 ± 2.157, $P = 0.02$, d.f. $= 10$). In contrast the decrease in TEE remained larger but was no more significant. As a result the numbers of meals eaten by a rat during HCD and/or the decrease in RQ in response to HF feeding are two parameters that discriminate FR and FS rats. Correlation analyses performed on these parameters showed that in 10 of the 12 rats the level of sensitivity to HFD was correlated to MN, IMI, MS, and the HFD-induced decrease in RQ (**Figure 2**). However, for all the correlations, the two same rats, the lowest and the highest BW gainers lay away from the regression lines suggesting that for these very resistant and sensitive individuals specific metabolic characteristics not shared by the other rats may be involved. If these two rats are taken into account only the correlation between BW gain and meal number remains significant ($P < 0.02$).

Predicting the CR/CS phenotype

The intensity of the bursts of activity was the parameter most significantly different between CR and CS rats. Classification of the rats according to the intensity of the bursts of activity required that one rat previously classified as CS that exhibited bursts of high intensity (3.97 U/100 g) had to be classified as potentially CR. This rat was the one with the second lowest adiposity level (13.59%). Recalculation of body composition of the two groups following intensity of activity bursts confirmed large differences in body fat between the two groups (37.48 ± 3.68 vs. 57.58 ± 3.51 g, $P = 0.003$), increased the differences in burst-intensity (3.764 ± 0.143 vs. 2.403 ± 0.119 U/100 g, $P = 0.00003$, d.f. $= 10$), and made overall activity significantly different between CR and CS rats (2.073 ± 0.130 vs. 1.608 ± 0.095 U/100 g, $P = 0.018$, d.f. $= 10$). In addition these differences under HCD remained significant during the 3-days of HFD. As a result, the intensity of bursts of activity and overall activity recorded in HCD fed rats are two parameters that discriminate CR and CS rats. Correlation analyses showed that a close quantitative relationship existed during HCD between the sensitivity to HCD and the intensity of the bursts of SPA. Mean SPA intensity and RQ were also potential predictors of CS, but were less accurate. No individuals appeared to diverge in the CR and CS rats (**Figure 3**). Interestingly, the level of carbohydrate sensitivity also correlated tightly with the speed of ingestion; however a large overlap between the speed of ingestion and the level of CS prevents speed of ingestion being a reliable predictive parameter (**Figure 3**).

DISCUSSION

First of all, it is important to discuss several limitations of this study. First, to avoid unnecessary sacrifice of animals, we assumed that a low number of animals could be used if an initial pre-selection of the rats by the breeder in a very large colony lead to initial important differences in their sensitivity to BW gain. This assumption seemed to work rather well because we were able to discriminate FR and FS rats that displayed highly significant differences in BW gain during HFD. In addition, the individuals ended the study with large differences in their adiposity (CR and CS rats). The fact remains however that the low number of animals reduces the statistical power available to discriminate between the various dietary CR/CS or FR/FS phenotypes. Additionally, because only four rats could be studied each week in the metabolic cages, sessions were staggered by 1–2 weeks, potentially contributing to variability in weight gain and body composition. We are rather

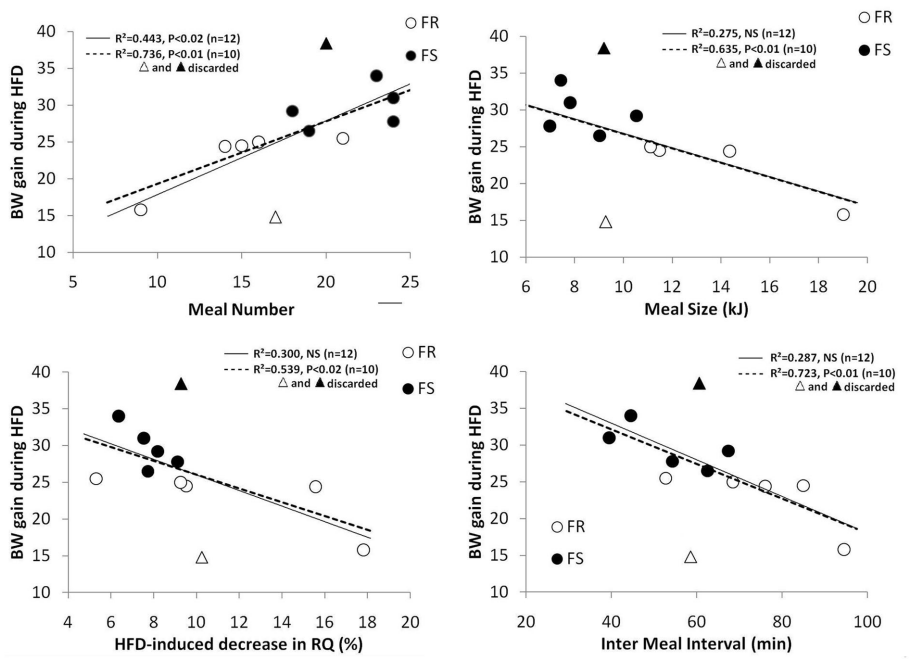


FIGURE 2 | Potential of MN, MS, and IMI measured under HCD to predict BW gain under HFD. The correlations have been computed with the 12 rats or after exclusion of the 2 outliers (triangles).

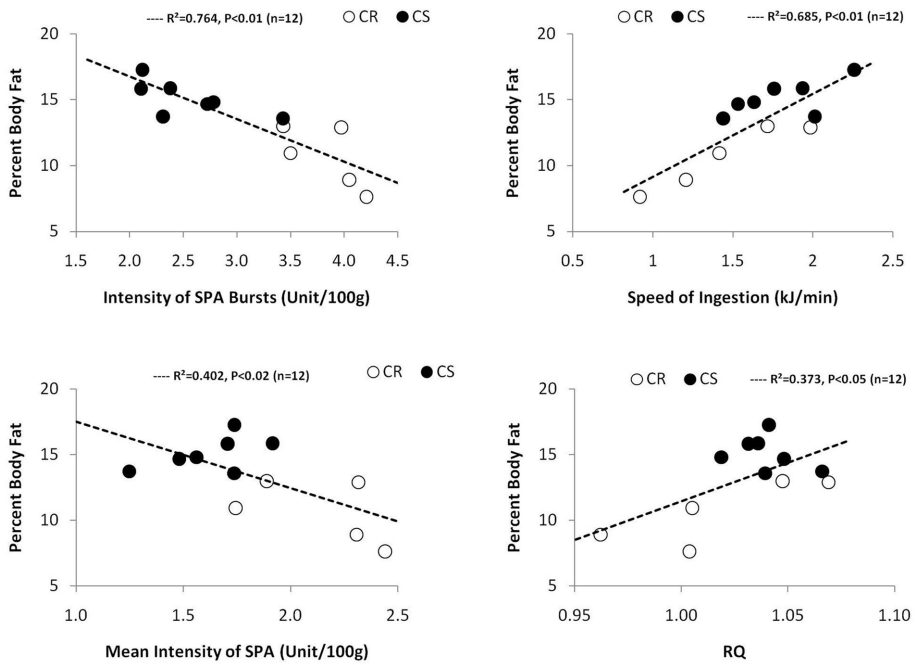


FIGURE 3 | Potential of intensity of SPA bursts, speed of ingestion, and mean intensity of SPA and RQ measured under HCD to predict development of body adiposity under HFD (all 12 rats included).

confident that this probably did not affect discrimination between FR and FS rats, the rate of BW gain decreasing only slowly over months. It is more difficult however to judge how this may have affected body composition. Encouragingly, in our hands body adiposity in male Wistar rats as measured by dissection analysis increases by less than 10% from 200 to 390 g (Even et al., 2001).

With respect to the CR/CS separation, this assumes that between-group differences in body fat accumulated during the 6-day exposure to HFD is a minor component of that accumulated over the entire study period. More generally, the grouping of the rats into CR/CS and FR/FS is limited by the lack of longitudinal monitoring of adipose mass throughout the various phases of this study. We intend to rectify this situation in the future by using MRI.

It should also be mentioned that the relatively short exposure of 6 days to HFD may be a limiting factor in discriminating between the FS and FR phenotypes. We would like to argue however that in most studies published hitherto FR and FS rats were separated from their BW gain and that much respected research groups (Pagliassotti et al., 1993; Commerford et al., 2001) have already shown that a 6-day period is long enough to discriminate between FR and FS subjects. On the other hand, the validity of the selection of the CR and CS rats after they were submitted to 6 days of HFD is indeed more disputable. We are, however, strongly convinced that the 6-day period of HFD did not significantly alter differentially the adiposity level of the CR and CS rats because the BW gain of these two groups during the HFD period was very similar (27.4 vs. 25.7 g). Also, as quoted above, the fundamentals of the selection between FR and FS rats used by most laboratories is that during HFD BW gain and body fat gain are highly correlated. In addition, recent unpublished MRI data obtained in our group indeed show that in our hands, the R^2 between body fat and BW gain during HFD is 0.767.

The goal of this study was to distinguish rats according to their sensitivity to HFD (FS vs. FR), but unexpected differences in body adiposity observed at the end of the study led us to also discriminate CR and CR rats and study sensitivity to HCD. We analyzed if differences in feeding, SPA and various components of energy metabolism could be predictive for these sensitivities to the diets. We observed that FS and CS rats exhibited different and specific metabolic and behavioral characteristics. Some of these characteristics were potentially reliable and non-invasive predictors to rapidly differentiate FS and CS rats while still lean and fed a usual HCD.

CHARACTERIZATION OF FS AND FR RATS

It is noteworthy that the FS rats did not gain more weight (therefore most probably not more fat) than the FR groups under HCD. Diets high in fat and consequently of higher energy density encourage food consumption (Poppitt, 1995), and accordingly in this study CI was larger during HFD than during HCD for all rats. However, FS rats did not eat significantly more food (g) than FR rats under either HFD or HCD. In contrast, FS rats ate significantly more meals under both diets but had smaller meals during the two first days of HFD. However, the reduction in meal size was only transient and vanished after 2–3 days of HFD. The fact that meal size was not larger in FS rats tends to contradict the idea that FS rats eat larger meals. However, such observations are usually made on rats that are already overweight after high fat feeding (Farley et al., 2003) which may confound pre-existing differences

in meal pattern with current obesity, metabolic, and endocrine adaptation to HFD.

That FS rats eat more meals than FR rats when fed a HCD has been reported previously in genetically selected FR and FS rats (Ricci and Levin, 2003; Cottone et al., 2007). Interestingly, these genetically selected rats seem similar to the outbred rats of this study since they were as lean as or even leaner than their FR counterparts when maintained under HCD. Meal fractioning has been proposed as a means to oppose obesity in humans (Cohn et al., 1965; Nicklas et al., 2001; Parks and McCrory, 2005) which contradicts the present observation that meal fractioning is associated with an increased sensitivity to obesity. In fact, meal fractioning does not seem to influence the predisposition to gain weight or fat. In FS rats, meal fractioning was higher under HCD when FS rats did not gain more weight or fat than FR rats, and was maintained under HFD when FS rats gained more weight. Therefore, meal fractioning is probably not responsible *per se* for the higher sensitivity to HF feeding in FS rats. Rather it may reveal some defect in the mechanisms controlling FI. For example, among potential defective signal(s), leptin production, or central sensitivity to leptin may be postulated. Indeed, central administration of leptin has been shown to reduce meal frequency in rats (Zorrilla et al., 2005) and to favor post-meal satiety (Montague et al., 1997; Chapelot et al., 2000; Westerterp-Plantenga et al., 2001). A defect in leptin sensitivity has also been reported in genetically selected FS rats (Levin et al., 2004; Clegg et al., 2005; Irani et al., 2007). Thus, measurement of meal number under HCD seems to be a criterion to easily and non-invasively separate FR and FS rats in a colony of young, non-obese unselected outbred rats, bearing in mind however that while there is indeed a good correlation, there is also overlap of individual values.

The fact that hyperphagia is a characteristic of FS rats is widely accepted and was even suggested to be a faithful predictor of sensitivity to obesity (Dourmashkin et al., 2006). Hyperphagia as the critical stimulus to increase BW gain in FS rats fed a HFD has also been suggested by the observation that FS rats have no increased inherent capacity for dietary fat retention (Commerford et al., 2001). However, in virtually every animal model of obesity in which hyperphagia is a characteristic feature, increased fat deposition has been shown to be independent of the increase in FI (Friedman, 1990, 1998). Therefore, if hyperphagia cannot be ruled out as a component of the sensitivity to HFD, at least for some of the FS rats, the present results confirm that increased FI is only one among several components responsible for increased weight gain of FS subjects under HFD.

Spontaneous physical activity is considered an important component involved in the resistance to HFD (Levine et al., 1999), but analysis of the various components of activity in FS and FR rats in this study did not reveal any significant difference, neither during HCD nor during HFD. In line with this absence of differences, genetically selected FS rats have not been shown to be less active than FR rats under HCD but to become less active only after overweight develops following long-term HFD (29 days; Novak et al., 2006). Thus, following the lack of a short-term effect of the HFD on SPA observed here, it is probable that the decrease in SPA after long-term HFD accompanies or results from, but is not responsible for, the gain in weight and the development of metabolic disturbances that progress during HFD.

Analysis of TEE and RQ suggests that the multiple pre-existing abnormalities reported in FS rats do not significantly affect these components of energy expenditure under HCD, a result that can be considered as normal since FS and FR rats maintained under the HCD ended the study with the same BW, LBM, carcass mass, fat mass, and adiposity. In contrast, during transition to the HF diet, TEE decreased more in FS rats while RQ tended to decrease less so that after 3 days of HFD RQ was higher in FS rats. These responses are obviously able to promote fat storage under HFD by saving energy and reducing fat oxidation. Therefore, studying the transition from HCD to HFD was able to reveal metabolic defects that were not visible under HCD. This suggests that processes specifically related to partitioning of dietary lipids between storage and oxidation are affected in FS rats during HFD but not HCD. Many potential mechanisms have been suggested in the literature, but one possible mechanism that we consider worth further investigation may be a defective lipid oxidation located specifically in muscles which progressively leads to fat accumulation in muscle and insulin resistance if HFD is continued (Galgani et al., 2008). To our knowledge, there has been no direct demonstration that this phenomenon pre-exists in still lean FS rats, but it has been reported that a significantly lower proportion of type I muscle fibers in the medial head of the gastrocnemius muscle of FS rats already before HFD may play a role in determining susceptibility to dietary obesity (Abou Mrad et al., 1992). Thus, changes in TEE and RQ during the first days of adaptation to HFD are potential parameters to discriminate between FR and FS rats.

CHARACTERIZATION OF CR AND CS RATS

Classification of CR and CS rats according to their body fat content at the end of the study showed that despite large differences in body fat, CS rats were only slightly, not significantly heavier than the CR ones, and had similar LBM and carcass mass. This may explain why this kind of rat had not been clearly distinguished, except, to our knowledge, in one publication (Dourmashkin et al., 2005) in which overfeeding and BW gain was boosted by a high content of saccharose in the diet which was not the case here. In addition we did not find in this study (but the number of rats was small) that CS rats were more sensitive to HFD than CR ones.

Contrary to what was observed between FR and FS rats, no differences were observed between CR and CS rats in any of the components of meal pattern under either HCD or HFD. In contrast, differences were observed at the level of SPA. CR rats developed bursts of SPA of higher intensity than the CS ones. The difference was particularly strong and significant during HCD, which is when the rats were fed the diet for which they exhibited a different sensitivity for fat deposition. The bursts of SPA correspond to the intensity of the mechanical work developed when the rats are active in contrast to the mean SPA that is the mean mechanical work developed during 24 h which includes resting periods. This component cannot be quantified with the usual red-beams device but requires a method able to quantitatively measure the forces that develop on the floor of the cage by force transducers located beneath the cage. We have extensive experience with this system and have been able to control that the intensity of the

activity signal tightly correlates to the energy expended in relation to muscular work (Even et al., 1994).

In humans, a reduced SPA is considered a trait of individuals sensitive to obesity and cardiovascular problems under HFD (Moore, 2000) and the increasingly sedentary habits associated with modern lifestyles are suggested to play an important role in the development of obesity (Prentice and Jebb, 1995). However, the influence on adiposity of the type of activity in which people engage during their daily life, or the way they perform standardized activities (walking, stair-climbing,) has not been systematically investigated. Here, we observed that the intensity of the bursts of SPA was very significantly lower in CS rats during HCD. This result suggests that rather than, or in conjunction with the absolute amount of activity, the type of activity, and specifically its intensity or its briskness may be involved in the resistance/sensitivity to fat deposition under HCD, possibly by influencing the control of energy metabolism and the aerobic/glycolytic capacities in muscles. For example, it was recently shown that rats with greater aerobic capacities were more active and also more resistant to HFD-induced obesity and responded differently to i.c.v. injections of orexin-A (Novak et al., 2010). Thus, activity and aerobic capacities in muscle are both important components of the response to HFD and we bring here preliminary data suggesting that this may well also be the case in rats that exhibit different sensitivities to HCD. On practical grounds, the amplitude of the differences in the bursts of activity between CS and CR rats makes this component of activity a potentially robust parameter to separate the two groups. It is in fact the only parameter for which we observed no overlap between CR and CS rats. This observation deserves further attention to confirm this phenomenon and further analyze how it is connected with the increased adiposity that develops in CS rats, in particular to what extent muscle metabolism is involved.

Measurements of TEE did not reveal differences between CR and CS rats. Comparison of CI with TEE showed that energy balance (EB) was not significantly different in the two groups under HC as well as under HF feeding. The increase in EB during HF feeding was mainly the result of an increase in CI while EE was unchanged, the amplitude of this phenomenon being comparable in the two groups. The fact that TEE does not increase (or only marginally so) after HF feeding has been reported in various studies (Dallosso and James, 1984; Schutz et al., 1989). However, it is interesting to note that despite not being significantly different, EB was steadily higher in CS than in CR rats. The difference was the largest (25%) under HC feeding. This was confirmed by differences between RQ values that were also larger in CS rats. The fact the EB and RQ values were not significantly different may be disappointing, but one must keep in mind that obesity develops very progressively as a result a very tiny daily differences that are necessarily very difficult to reveal by direct measurement of energy expenditure. Differences in RQ have also been reported under HF feeding between FS and FR (Chang et al., 1990), and the authors suggested that the capacity to adjust nutrient oxidation to nutrient intake was a major mechanism underlying the sensitivity to HF feeding. This is obviously a potential mechanism, and it seems logical that in the CS rats of the present study, differences in RQ may be larger under HCD than under HFD.

CONCLUSION

The study reported here was devoted to revealing parameters giving early indications of a potential sensitivity to increased adiposity under HFD and show that rats differ not only in their sensitivity to HFD but also in their sensitivity to HCD, this latter sensitivity being more difficult to reveal because it induces only small differences in BW gain.

One major point to emphasize is that we did not eliminate any individuals from the group of 12 rats that were introduced in the study, but rather asked the breeder to provide us heterogeneous rats from their colony. Despite the initial disappointing observation that the difference in BW between the light and heavy rats was small, this approach probably helped to discriminate FS and FR rats as well as CR and CS rats within a small group and to reveal significant correlations between various metabolic and behavioral parameters and the level of sensitivity to HFD as well as HCD.

The best predictors of the dietary sensitivity of the rats are behavioral parameters related to feeding behavior, mainly SPA. RQ is also a potential predictor, but the greater difficulty to get a precise and reproducible measure together with the overlap of individual values makes RQ useful only for discrimination between extreme individuals.

ACKNOWLEDGMENTS

This study was funded by grants from the *Institut national de la rechercheagronomique* (French National Institute for Agricultural Research, INRA), *Centre National de la Recherche Scientifique* (French National Center for Scientific Research, CNRS), and the "*Institut Benjamin Delessert*." This work was primarily that of Patrick Christian Even, who was the main instigator at all stages. Gilles Fromentin and Daniel Tomé helped conceive the work, Catherine Chaumontet and Dalila Azzout aided in body composition measurement, Nachiket A. Nadkarni assisted in data analysis, Nachiket A. Nadkarni and Daniel Tomé contributed to writing this manuscript. The authors would like to thank AngéliqueSimonin and JulienPiedcoq for their help in the management of the rats and their participation in carcass analyses.

REFERENCES

Abou Mrad, J., Yakubu, F., Lin, D., Peters, J. C., Atkinson, J. B., and Hill, J. O. (1992). Skeletal muscle composition in dietary obesity-susceptible and dietary obesity-resistant rats. *Am. J. Physiol.* 262, R684–R688.

Chang, S., Graham, B., Yakubu, F., Lin, D., Peters, J. C., and Hill, J. O. (1990). Metabolic differences between obesity-prone and obesity-resistant rats. *Am. J. Physiol.* 259, R1103–R1110.

Chapelot, D., Aubert, R., Marmonier, C., Chabert, M., and Louis-Sylvestre, J. (2000). An endocrine and metabolic definition of the intermeal interval in humans: evidence for a role of leptin on the prandial pattern through fatty acid disposal. *Am. J. Clin. Nutr.* 72, 421–431.

Clegg, D. J., Benoit, S. C., Reed, J. A., Woods, S. C., Dunn-Meynell, A., and Levin, B. E. (2005). Reduced anorexic effects of insulin in obesity-prone rats fed a moderate-fat diet. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 288, R981–R986.

Cohn, C., Joseph, D., Bell, L., and Allweiss, M. D. (1965). Studies on the effects of feeding frequency and dietary composition on fat deposition. *Ann. N. Y. Acad. Sci.* 131, 507–518.

Commerford, S. R., Pagliassotti, M. J., Melby, C. L., Wei, Y., and Hill, J. O. (2001). Inherent capacity for lipogenesis or dietary fat retention is not

increased in obesity-prone rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 280, R1680–R1687.

Cottone, P., Sabino, V., Nagy, T. R., Coscina, D. V., and Zorrilla, E. P. (2007). Feeding microstructure in diet-induced obesity susceptible versus resistant rats: central effects of urocortin 2. *J. Physiol. (Lond.)* 583, 487–504.

Dallosso, H. M., and James, W. P. (1984). Whole-body calorimetry studies in adult men. 1. The effect of fat overfeeding on 24 h energy expenditure. *Br. J. Nutr.* 52, 49–64.

Dourmashkin, J. T., Chang, G. Q., Gayles, E. C., Hill, J. O., Fried, S. K., Julien, C., and Leibowitz, S. F. (2005). Different forms of obesity as a function of diet composition. *Int. J. Obes. (Lond.)* 29, 1368–1378.

Dourmashkin, J. T., Chang, G. Q., Hill, J. O., Gayles, E. C., Fried, S. K., and Leibowitz, S. F. (2006). Model for predicting and phenotyping at normal weight the long-term propensity for obesity in Sprague-Dawley rats. *Physiol. Behav.* 87, 666–678.

Even, P. C., Mokhtarian, A., and Pele, A. (1994). Practical aspects of indirect calorimetry in laboratory animals. *Neurosci. Biobehav. Rev.* 18, 435–447.

Even, P. C., Rolland, V., Roseau, S., Bouthegourd, J. C., and Tome, D. (2001). Prediction of basal metabolism from organ size in the rat: relationship to strain, feeding, age, and obesity. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 280, R1887–R1896.

Farley, C., Cook, J. A., Spar, B. D., Austin, T. M., and Kowalski, T. J. (2003). Meal pattern analysis of diet-induced obesity in susceptible and resistant rats. *Obes. Res.* 11, 845–851.

Ferrannini, E. (1988). The theoretical bases of indirect calorimetry: a review. *Metab. Clin. Exp.* 37, 287–301.

Flatt, J. P. (1987). The difference in the storage capacities for carbohydrate and for fat, and its implications in the regulation of body weight. *Ann. N. Y. Acad. Sci.* 499, 104–123.

Flatt, J. P. (1988). Importance of nutrient balance in body weight regulation. *Diabetes Metab. Rev.* 4, 571–581.

Friedman, M. I. (1990). Body fat and the metabolic control of food intake. *Int. J. Obes.* 14(Suppl. 3), 53–66; discussion 66–57.

Friedman, M. I. (1998). Fuel partitioning and food intake. *Am. J. Clin. Nutr.* 67, 513S–518S.

Galgani, J. E., Moro, C., and Ravussin, E. (2008). Metabolic flexibility and insulin resistance. *Am. J. Physiol. Endocrinol. Metab.* 295, E1009–E1017.

Irani, B. G., Dunn-Meynell, A. A., and Levin, B. E. (2007). Altered hypothalamic leptin, insulin, and melanocortin binding associated with moderate-fat diet and predisposition to obesity. *Endocrinology* 148, 310–316.

Ji, H., and Friedman, M. I. (2003). Fasting plasma triglyceride levels and fat oxidation predict dietary obesity in rats. *Physiol. Behav.* 78, 767–772.

Lacroix, M., Gaudichon, C., Martin, A., Morens, C., Mathe, V., Tome, D., and Huneau, J. F. (2004). A long-term high-protein diet markedly reduces adipose tissue without major side effects in Wistar male rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 287, R934–R942.

Layman, D. K., Evans, E. M., Erickson, D., Seyler, J., Weber, J., Bagshaw, D., Griel, A., Psota, T., and Kris-Etherton, P. (2009). A moderate-protein diet produces sustained weight loss and long-term changes in body composition and blood lipids in obese adults. *J. Nutr.* 139, 514–521.

Levin, B. E., Dunn-Meynell, A. A., Balkan, B., and Keesey, R. E. (1997). Selective breeding for diet-induced obesity and resistance in Sprague-Dawley rats. *Am. J. Physiol.* 273, R725–R730.

Levin, B. E., Dunn-Meynell, A. A., and Banks, W. A. (2004). Obesity-prone rats have normal blood-brain barrier transport but defective central leptinsignaling before obesity onset. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 286, R143–R150.

Levine, J. A., Eberhardt, N. L., and Jensen, M. D. (1999). Role of nonexercise activity thermogenesis in resistance to fat gain in humans. *Science* 283, 212–214.

Montague, C. T., Farooqi, I. S., Whitehead, J. P., Soos, M. A., Rau, H., Wareham, N. J., Sewter, C. P., Digby, J. E., Mohammed, S. N., Hurst, J. A., Cheetham, C. H., Earley, A. R., Barnett, A. H., Prins, J. B., and O'Rahilly, S. (1997). Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature* 387, 903–908.

Moore, M. S. (2000). Interactions between physical activity and diet in the regulation of body weight. *Proc. Nutr. Soc.* 59, 193–198.

Neel, J. V. (1999). The "thrifty genotype" in 1998. *Nutr. Rev.* 57, S2–S9.

Nicklas, T. A., Baranowski, T., Cullen, K. W., and Berenson, G. (2001). Eating patterns, dietary quality and obesity. *J. Am. Coll. Nutr.* 20, 599–608.

Novak, C. M., Escande, C., Burghardt, P. R., Zhang, M., Barbosa, M. T., Chini, E. N., Britton, S. L., Koch, L. G., Akil, H., and Levine, J. A. (2010). Spontaneous activity, economy of activity, and resistance to diet-induced obesity in rats bred for high intrinsic aerobic capacity. *Horm. Behav.* 58, 355–367.

Novak, C. M., Kotz, C. M., and Levine, J. A. (2006). Central orexin sensitivity, physical activity, and obesity in diet-induced obese and diet-resistant rats. *Am. J. Physiol. Endocrinol. Metab.* 290, E396–E403.

Pagliassotti, M. J., Shahrokhi, K. A., and Hill, J. O. (1993). Skeletal muscle glucose metabolism in obesity-prone and obesity-resistant rats. *Am. J. Physiol.* 264, R1224–R1228.

Parks, E. J., and McCrory, M. A. (2005). When to eat and how often? *Am. J. Clin. Nutr.* 81, 3–4.

Pichon, L., Huneau, J. F., Fromentin, G., and Tome, D. (2006). A high-protein, high-fat, carbohydrate-free diet reduces energy intake, hepatic lipogenesis, and adiposity in rats. *J. Nutr.* 136, 1256–1260.

Poppitt, S. D. (1995). Energy density of diets and obesity. *Int. J. Obes. Relat. Metab. Disord.* 19(Suppl. 5), S20–S26.

Prentice, A. M., Hennig, B. J., and Fulford, A. J. (2008). Evolutionary origins of the obesity epidemic: natural selection of thrifty genes or genetic drift following predation release? *Int. J. Obes. (Lond.)* 32, 1607–1610.

Prentice, A. M., and Jebb, S. A. (1995). Obesity in Britain: gluttony or sloth? *BMJ* 311, 437–439.

Ricci, M. R., and Levin, B. E. (2003). Ontogeny of diet-induced obesity in selectively bred Sprague-Dawley rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 285, R610–R618.

Schutz, Y., Flatt, J. P., and Jequier, E. (1989). Failure of dietary fat intake to promote fat oxidation: a factor favoring the development of obesity. *Am. J. Clin. Nutr.* 50, 307–314.

Speakman, J. R. (2007). Anonadaptive scenario explaining the genetic predisposition to obesity: the "predation release" hypothesis. *Cell Metab.* 6, 5–12.

Stoger, R. (2008). The thrifty epigenotype: an acquired and heritable predisposition for obesity and diabetes? *Bioessays* 30, 156–166.

Westerterp-Plantenga, M. S., Saris, W. H., Hukshorn, C. J., and Campfield, L. A. (2001). Effects of weekly administration of pegylated recombinant human OB protein on appetite profile and energy metabolism in obese men. *Am. J. Clin. Nutr.* 74, 426–434.

Zorrilla, E. P., Inoue, K., Valdez, G. R., Tabarin, A., and Koob, G. F. (2005). Leptin and post-prandial satiety: acute central leptin more potently reduces meal frequency than meal size in the rat. *Psychopharmacology (Berl.)* 177, 324–335.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 21 September 2011; accepted: 22 November 2011; published online: 16 December 2011.

Citation: Even PC, Nadkarni NA, Chaumontet C, Azzout-Marniche D, Fromentin G and Tomé D (2011) Identification of behavioral and metabolic factors predicting adiposity sensitivity to both high fat and high carbohydrate diets in rats. Front. Physio. ***2****:96. doi: 10.3389/fphys.2011.00096*

This article was submitted to Frontiers in Integrative Physiology, a specialty of Frontiers in Physiology.