Chapter 12

Diet-Induced Thermogenesis: Principles and Pitfalls

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Abstract

Concerning diet-induced thermogenesis, methodological issues relate mainly to the interpretation of measurements, rather than to the technical methodology as such. In the following, we point to a series of issues where the analysis often suggests the occurrence of UCP1-related diet-induced thermogenesis but where the observations are often the *consequences* of a process that has induced leanness rather than being the *cause* of them. We particularly emphasize the necessity of focusing on the total organism when interpreting biochemical and molecular data, where the concept of total tissue values rather than relative data better reflects physiologically important alterations. We stress the importance of performing experiments at thermoneutrality in order to obtain clinically relevant data and stress that true thermogenic agents may be overlooked if this is not done.

Key words UCP1, Thermogenesis, Diet-induced thermogenesis, Metabolic efficiency

1 Introduction

A basic problem (or perhaps *the* basic problem) when studying diet-induced thermogenesis is that it is uncertain whether the phenomenon exists—in part because it is difficult to define experimentally.

The basic concept is that an organism, given a series of objective factors, would be expected to utilize a certain amount of energy (calories, joules) per day. Theoretically, any calories in excess of this amount should then be accumulated and stored as lipids. The point of diet-induced thermogenesis would be that while such an extra intake of calories does result in some accumulation of lipid, the amount of lipid accumulated would be lower than calculated. The extra energy that has disappeared must then necessarily have been dissipated as heat ("gone up in smoke"). We would then refer to this extra heat production as diet-induced thermogenesis. To some extent, the existence of such a process may seem so farfetched that it

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could be considered a myth—as indeed has been said, more than once [1, 2].

2 Principles

2.1 Obligatory Diet-Induced Thermogenesis

One problem with the analysis is that a certain fraction of the energy content of food items will necessarily always be used for digestion, anabolic synthesis, etc. This is what is referred to as obligatory diet-induced thermogenesis (or the thermal effect of feeding, TEF) and this is thus an additional component above basal metabolic rate. Thus, a measurable amount of thermogenesis accompanies food intake, and the feeding period is also generally associated with the time of the day with a higher general activity. This means both that the mere observation of a higher rate of thermogenesis (metabolic rate) at feeding time is not a demonstration of the existence of diet-induced thermogenesis. A further problem is that there are no confirmed and accepted values for the percentage of food energy that is used for obligatory thermogenesis. Standard literature values for this obligatory thermogenesis are for carbohydrates 5–10%, for lipids 0–3% and for protein 20–30% (as summarized by [3]). Additionally, with the method used to obtain these values, it cannot be excluded that the thermogenesis observed also includes a component from "true" diet-induced thermogenesis, i.e., facultative diet-induced thermogenesis.

2.2 Facultative Adaptive Diet-Induced Thermogenesis

Facultative adaptive diet-induced thermogenesis is thus that fraction of total "diet-induced thermogenesis" the existence of which is doubted and where the need for further experimentation exists. *Facultative* implies that it exists in addition to the obligatory fraction and that it only exists under certain circumstances; adaptive means that it requires chronic exposure to the inducing stimuli in order for the capacity to develop.

A principle for establishing the existence of diet-induced thermogenesis is summarized in Fig. 1. As seen, the only convincing way to demonstrate its existence is to remove the source/mechanism; this will thus result in increased lipid accumulation, given unchanged energy intake.

2.3 Where Is Diet-Induced Thermogenesis Located?

Three questions are thus closely connected: does diet-induced thermogenesis exist? what is the mechanism? where is it located? For the last 40 years [4], the suggested answer has been that diet-induced thermogenesis is located in brown adipose tissue, and that the adaptive character of the phenomenon is due to recruitment of the tissue. More specifically, the suggestion has been that diet-induced thermogenesis is the result of the amount and activity of UCP1 in brown adipose tissue, as well as a consequence of the browning of other tissues, primarily the browning of subcutaneous (mainly inguinal) adipose tissue, leading to increased amounts of (brite/beige) adipose tissues.

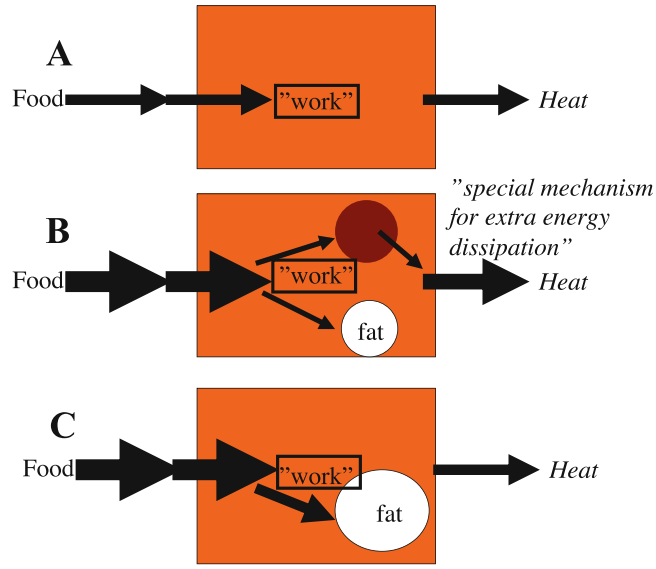

Fig. 1 The principle for detection of facultative diet-induced thermogenesis. In a theoretical equilibrium situation, the energy content of the ingested food is identical to that which is necessary to produce the energy used for the organism's "work"; the work would be the combined cost of the organism's work on the surroundings and the work needed to keep bodily functions in equilibrium (**a**). Under conditions where the food energy intake is higher than that necessary for "work," the extra energy could be divided between that combusted in a "special mechanism for extra energy dissipation" (diet-induced thermogenesis) and that accumulated in adipose tissue (**b**). The existence of such a mechanism of diet-induced thermogenesis would be revealed if the mechanism was suppressed: more adipose tissue should then result from the same "extra" energy intake (**c**)

A direct consequence of this suggestion is that mice in which diet-induced thermogenesis is recruited should possess more UCP1, and that, with identical food intake, mice without UCP1 should become more obese, given the paradigm sketched in Fig. 1c. Indeed, a positive association between states expected to induce diet-induced thermogenesis and the amount of UCP1 has been observed [5–8]. Correspondingly, the absence of BAT/UCP1 has been observed to be associated with the development of modest obesity [5–11]—but here the outcome is not consistent, and several reports see no fattening effects of UCP1 ablation [12–14]. To some extent, these discrepancies are explainable by the different environmental temperatures at which the mice are living—since, at environmental temperatures below thermoneutrality, the necessity for extra heat production derived from cold-induced classical nonshivering thermogenesis can be replaced by any diet-induced thermogenesis that is occurring, and no effects will be observable (see below)—but this is not the case for all

investigations (e.g., [14]). The absence of UCP1 can in itself lead to secondary alterations in the tissue [15]; however, diet-induced thermogenesis experiments should be performed at thermoneutrality and the secondary effects of UCP1 ablation under these conditions are very modest or non-existent.

Thus, the existence of diet-induced thermogenesis is still not unequivocally established, and particularly not whether it is totally attributable to brown adipose tissue activity.

2.4 Why Would Diet-Induced Thermogenesis Exist?

One reason that diet-induced thermogenesis has been (or is) considered to be a myth is the—at least apparent—implausibility that nature would evolve processes where food (that would normally be considered scarce) would be consumed merely to be combusted. It may not be as perplexing as it sounds. One possibility, originally advocated by Michael Stock [16] in his protein leverage hpothesis, is that diet-induced thermogenesis has developed as a means to allow animals to obtain sufficient amounts of valuable constituents in the food, even though the content of these in the food may be low. This "sifting" hypothesis would then mean that the animal could overeat foods with, e.g., a low protein content in order to obtain sufficient essential amino-acids, without storing excess amounts of lipid and thus becoming an easy object of prey. Examination of the validity of this hypothesis is still ongoing.

2.5 The Experimental Models

Basically, two experimental models are used to investigate the existence and possible properties of diet-induced thermogenesis. In the *hedonic model*, some mice are exposed to a palatable diet, others to normal, rather boring, chow. The palatable diet can be varied, as in early versions of a "cafeteria feeding" [4] or a somewhat simpler composition [8] or simply one of the high-fat/high-sugar diets from a commercial supplier such as Research Diets (these diets are often described as "high-fat," but in addition to the high energy content in the form of lipid, they generally also contain a high content of carbohydrate in the form of sugar, yielding a food that is attractive for both rodent and human taste). The mice tend to eat significantly more of the hedonic diets, at least initially, and certain mouse strains, particularly the popular C57BL/6J ("Black 6") strain, will gain a lot of body fat, increasing from some 25 g total body weight with some 3 g lipid to about 50 g body weight with about 20 g lipid. The issue is thus whether these obese mice have recruited diet-induced thermogenesis, i.e., whether, even though they are obese, they have attained a higher amount of UCP1, a higher capacity for diet-induced thermogenesis.

In the *slimming model*, mice are made obese, often exactly as in the hedonic model with free access to a high-fat diet. As can be understood, this in itself may be a problematic avenue for examining "extra" diet-induced thermogenesis since these mice may already have acquired an increased amount of UCP1 and a higher

capacity for diet-inducecd thermogenesis. Some of the mice are then exposed to a food component, often added to the diet, to examine whether this food component can induce extra thermogenesis that would lead to body weight reduction in the mice.

The methods for examination of these models would appear straightforward, but experience shows that particularly the obesity that is induced in both models makes the interpretation of the results difficult, often leading to claims that diet-induced thermogenesis has been demonstrated under conditions where this is not the case. We will point to some of these pitfalls in the following.

The underlying caveat is thus that if an agent (food substance etc.) induces a weight-reducing process, the changes observed in brown (and other) adipose tissues may be interpreted to indicate that UCP1-dependent diet-induced thermogenesis has been induced and that this is the cause of the weight reduction—whereas these changes may be the consequences of the reduction in body weight (obesity) rather than the cause. What, in these cases, causes the reduction in body weight is not evident, but it must be emphasized that even very small changes in food intake (<5%) can with time lead to substantial weight loss. Thus, even a small decrease in the attractiveness of the food may have this effect and thus explain the decrease in body weight without a need to involve a thermogenic process. What we discuss below are thus the parameters that have been evoked to establish the occurrence of diet-induced thermogenesis in these two models. As will be seen, teh establishment of these parameters may be associated with several pitfalls.

3 Possible Pitfalls

For examination and characterization of all issues related to diet-induced thermogenesis, the main way to think should be integrative and organismal. Concerning measurements that are intended to characterize the energy balance of the mouse as a whole (and here we will write "mouse" for simplicity, but the issues are relevant for studies of all mammals), they should all be expressed per total mouse. This is often not the case.

Concerning measurements that refer to brown adipose tissue in this context, it is very important that the same integral, whole animal point of view is maintained. Traditionally, this is rarely done—but, as specified below, interpretations can be radically different when data are expressed using the integrative view of the entire brown adipose tissue—and thus for the entire animal - versus only for a sample of brown adipose tissue. This is because what matters to the mouse is the capacity of the entire tissue, not that in a small sample of it.

Concerning especially the weight-loss model, the standard picture is often that a mouse is made obese by a standard high-fat diet

and then is treated with some (food-related) item, normally added directly to the food. During this treatment, the mouse loses weight. The leaner mice are then examined and shown to present with an (apparently) increased metabolic rate, an (apparently) activated/recruited brown or brite/beige adipose tissue, often accompanied by an ameliorated diabetic state. These changes are then discussed as being causative of the amelioration in health, through enhanced diet-induced thermogenesis that, in its turn, is caused by the activated adipose tissues. The important issue is the cause-versus-effect issue. Thus, the issue is whether the signs reported of increased thermogenesis and BAT recruitment are causative of the weight-loss process—or whether the data presented rather are the result of a weight-loss process and thus *due to* a decrease in obesity rather than *causing* the decrease in obesity. The pitfalls discussed here are pitfalls that tend to favor an interpretation indicating causative effects of brown and brite/beige adipose tissue, even if the data actually indicate that the alterations in these tissues rather are secondary to the loss of body fat. To comprehend the scientific and translational significance of data relating to diet-induced thermogenesis, it is thus important that these pitfalls are avoided.

3.1 Metabolic Characterization

3.1.1 Perform Experiments at Thermoneutrality

Mice are routinely kept at temperatures that we—as clothed, physically active humans—consider comfortable for us; this means normally 18–22 °C. However, this temperature is below the thermoneutral temperature for mice; the thermoneutral temperature for normal adult mice is about 30 °C. This is also the environmental temperature a mouse will choose if it is given a choice [17]. Below this temperature, the mouse uses significant amounts of energy to defend its body temperature; the increase in metabolism from 30 to 20 °C is some 50–100% [17]. Therefore, to examine mice for the existence of diet-induced thermogenesis at "normal" animal house temperatures induces risks for two kinds of misleading results: the false positives and—worse—the false negatives.

Concerning the *false positives*, these can be caused by a potentially "weight-reducing compound" that has as its effect that the heat insulation capacity of the mouse is diminished. If insulation is decreased, the mouse will lose more heat at a given temperature, and more energy will have to be used to compensate for this, thus inducing weight loss (*see* Fig. 2a). This will also secondarily cause activation of BAT, as discussed in detail elsewhere for other "browning" agents [18]. Although such a scenario is similar in several respects to that which would be expected of true diet-induced thermogenesis, the thermogenesis is not diet-induced and can be mimicked, e.g., by shaving the mouse [19].

The *false negatives* are probably more scientifically disturbing. In this scenario, a genuine inducer of diet-induced thermogenesis may exist that, if examined at thermoneutrality, would indeed

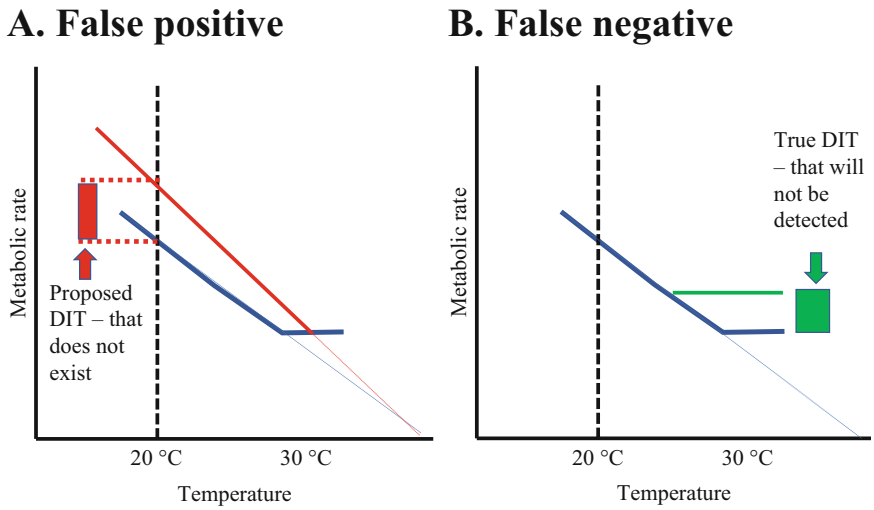

Fig. 2 The background for false positive and false negative results when identifying agents proposed to induce diet-induced thermogenesis. Below thermoneutrality ("30 °C"), mouse metabolism increases linearly with decreasing temperature. As seen in A, (supposedly) diet-induced thermogenesis-inducing agent in reality induces a reduced insulation (red line), a (non-existant) diet-induced thermogenesis may be claimed. As seen in B, a true increase in metabolic rate observed at thermoneutrality (green line) will disappear when the mouse is examined (housed) below thermoneutrality

induce an increase in metabolic rate. However, as seen in Fig. 2b, if the examination is performed at subthermoneutral temperatures, the mouse would simply use this extra heat production as part of the heat needed to defend its body temperature, and the genuine increase would not be observable. Thus, given the conditions under which most examinations of diet-induced thermogenesis are presently performed, it is very likely that potential candidates that are genuine inducers of diet-induced thermogenesis have been missed. Their presence would not affect metabolic rate at normal animal house temperatures, and there would be no slimming effect.

Additionally, for unknown reasons, particularly concerning the effect of UCP1 ablation, ambient temperatures below thermoneutrality may qualitatively affect the outcome. Under conditions of high-fat diet treatment, the absence of UCP1—that would generally be expected to enhance obesity at thermoneutrality [5–8]—no longer does so; rather the absence seems to protect against diet-induced obesity [13] (our unpubl. obs.) [20]. Although suggestions have been made [20], there is presently no full understanding of this phenomenon.

3.1.2 Food Intake Measurements Are Essential but Difficult to Perform

"Weight-reducing agents" that are expected to work through inducing or enhancing diet-induced thermogenesis are often added directly to the food. It is therefore possible that they may affect food intake by slightly affecting, e.g., the taste or texture of the food; similarly, such agents could slightly affect appetite in

other ways. The problem in identifying these confounders is due to the fact that very small decreases in food intake—2–4%—will, with time, lead to a substantial loss of body weight, at least when calculated theoretically. Such small changes are in general difficult to substantiate. Since food intake is often measured very crudely (the weight of the food holder at the start minus that at the end plus what is left on the bottom of the cage), food intake measurements are prone to large data variance. A high variance makes it even more difficult to establish statistically significant effects on food intake. Occasionally, when food intake measurements indicating apparent differences between control and treated mice are shown—but due to the high variance, the difference does not reach statistical significance—the authors conclude that there therefore *is* no difference; we find it likely that in some instances this conclusion is a statistical "beta"-mistake: that the statistical analysis fails to recognize a difference that is there (or that the experiment is "underpowered" given the variation in the food intake measurements). We consider that minor differences in food intake more often than is generally believed may cause weight reduction, but we see no simple way to improve the quality of these measurements.

3.1.3 Indirect Calorimetry Chambers Are Stressful

The most direct way of showing diet-induced thermogenesis would seem to be to use (indirect) calorimetry chambers, where thermogenesis is determined by measuring oxygen utilization and carbon dioxide production. However, despite the fact that the environment in the calorimetry chambers should not be different from that which the mice experience in daily life, the general observation is that transfer of the mice into these chambers disturbs the mice, and even the control mice often, e.g., lose body weight, in reality invalidating the data obtained. The only solution is to let the mice stay in the chambers for a prolonged period of time (several days minimally) before the relevant measurements are made.

3.1.4 Do Not Divide by Body Weight

This is one of the most important pitfalls in issues relating to diet-induced thermogenesis.

The outcome of measurements in indirect calorimetry chambers is principally a value for heat production (oxygen consumption) per mouse. However, unfortunately, the manufacturers of these instruments generally set the program to present the main result as thermogenesis per g body weight, and this is also the data that most core facilities deliver. This practice is undoubtedly the largest problem within the field of diet-induced thermogenesis and means that a plethora of agents have been ascribed abilities to increase diet-induced thermogenesis—despite the fact that in very many cases, no such increase exists.

There has been a long tradition in metabolic studies to express measured quantities, such a metabolic rate, per kg body weight

(and in certain contexts, per (body weight)$^{0.67}$ or (body weight)$^{0.75}$). The simple rule in diet-induced thermogenesis studies is: *do not do this*. The main reason is the nature of the weight changes in obesity/diet-induced thermogenesis studies.

If studies are performed where, e.g., a large variety of animals are captured in nature and parameters should be compared, it is natural to compensate in a simple way for body size. This is similarly the case if comparisons are made between animals of different species with very different body sizes. In some way, the body composition in these conditions is expected to largely harmonize with size and this can be accounted for by dividing by size (g body weight). However, this is not the case in obesity- and diet-induced thermogenesis-related studies. In these studies, the control mice and the mice that subsequently become obese are initially identical and in metabolic terms may be said to consist of lean mass and fat mass, as can be detected and distinguished with magnetic resonance imaging (MRI) or dual-energy X-ray absorptiometry (DEXA). The lean mass is the fraction that is metabolically active; in reality it is mainly water and protein. The fat mass, determined in this way, consists totally of inert lipid, just like a packet of butter, and is therefore, as such, totally unable to contribute actively to metabolism (Fig. 3a). It is thus important to emphasize that fat mass is not "adipose tissue mass" that obviously consists of other components than pure lipid, but these components are included in the lean mass.

It is advantageous to start obesity-related experiments when the mice are about 12 weeks old, because at that time the mice are fully adult, and the body weight does not change markedly during the following weeks. The body weight of a male C57B/6 mouse will be about 25 g and the lean mass some 22 g thereof, with thus about 3 g fat mass (Fig. 3a). When diet-induced obesity has been induced, the body weight will typically have increased to about 46 g, of which fat mass will be 20 g and lean mass 26 g (Fig. 3b). The increase in fat mass is thus some 470% but the increase in lean mass only some 18%; this increase in lean mass represents mainly the non-fat part of the expanding adipose tissue. Some 10–20% of adipose tissue consists of non-fat; thus, an increase in body weight of 21 g due to an increase in adipose tissue will consist of 17 g fat and 4 g lean mass.

In metabolic terms, the obese mouse is therefore practically identical to the non-obese mouse, except that it is carrying a rucksack of totally inert lipid. Just as we would not imagine that our "real" weight increased by carrying a rucksack, nor has the "metabolic" weight of the mouse really increased either (this does not mean, of course, that there may not be other effects of the rucksack/obesity).

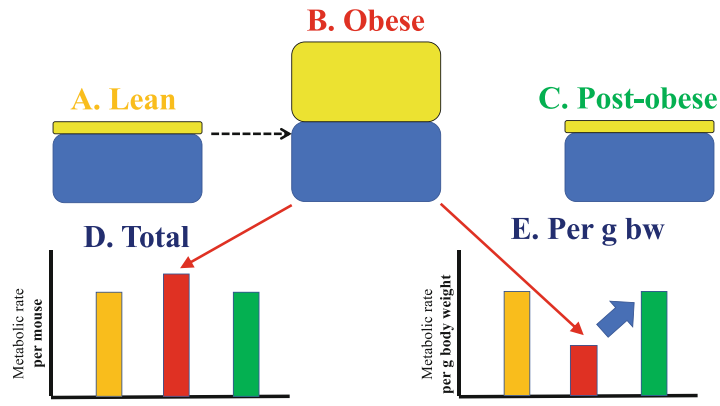

Fig. 3 The misleading effect of dividing by body weight for analysis of diet-induced thermogenesis. (**a**) The body composition of a lean (normal, control) mouse; blue: lean mass; yellow: fat mass. (**b**) The body composition of a diet-induced obese mouse; note that the lean mass is slightly larger due to the lean mass component of the extra adipose tissue. (**c**) The body composition of a post-obese mouse. (**d**) The metabolic rate in the 3 conditions, expressed per mouse. Note that the rate is slightly higher in the obese mouse (red), due to the slightly higher lean mass. (**e**) The metabolic rate in the 3 "?>conditions, expressed per g mouse. Note that the rate is much lower in the obese mouse (red), due to the much higher total mouse mass. Thus, the result of the study is very different if formulated per mouse or per g mouse. Particularly, the apparent impressive increase in metabolic rate expressed per g mouse seen in the post-obese versus the obese mice (heavy blue arrow) is often stated as indicating a recruitment of diet-induced thermogenesis; in reality it is often only an effect of the divisor

If the metabolic rates of the control and the obese mouse are now measured, the probable result is that the metabolic rate is some 18% higher in the obese mouse, due to its higher amount of lean mass (Fig. 3c). However, if the misleading convention of dividing by body weight is used, the outcome is then very different and mathematically unavoidable: obese mice appear to have a lower metabolic rate than “lean” mice, and the obesity is thus “explained” by this apparent decrease in metabolic rate. Correspondingly, any agent that reduces the body weight towards that of the lean mice will apparently be increased;"?>will result in an apparent increse in metabolic rate; the slimming effect will thus be “explained” by diet-induced thermogenesis that does not really exist.

The misleading interpretations that result from dividing by body weight in this type of research have repeatedly been discussed [21–25], but routine representations in studies of diet-induced thermogenesis still overwhelmingly show metabolic rates expressed per kg body weight, and the number of suggested proposed inducers of diet-induced thermogenesis therefore vastly exceeds the number of true ones.

Our conclusion is thus to express metabolic rate *per mouse* if energy balance is discussed (or if you do not have access to MRI instruments). Data may be expressed per g *lean* mass if you have access to the lean mass data and search for a cause of increased metabolic rate that is not explainable by the small increase in lean mass in obese mice. The absence of an ability to obtain data for lean mass should *not* be an excuse for dividing by body weight.

An ancova-analysis is sometimes suggested [24] and may be relevant in certain conditions. This is particularly the case if the population is very varied, based on other factors than obesity (sex, age, time of year, ambient temperature, habitat). Ancova-analysis in that case can reveal the contribution of the different factors. However, in the type of "preclinical" experiments that dominate within diet-induced thermogenesis research, other such factors are practically absent, and the necessity of ancova is low. And, as pointed out by Arch et al.: "The simple message is 'use ANCOVA to analyse studies of treatment effects on EE. In those cases where ANCOVA is not appropriate (low within-group variance relative to treatment effect on mass and EE) at the very least give EE per animal as well as transformed data (preferably divided by lean body mass and not divided by whole body mass or metabolic mass). Always make comparisons between intake and expenditure using equivalent units (mass corrected or absolute)" [26].

Thus, the title of this section is of such importance for the true outcome of diet-induced thermogenesis studies that we will repeat it as the conclusion of this section: in studies of diet-induced thermogenesis and similar: ***Do not divide by body weight!***

3.1.5 In an Equation, the Two Sides Must Be Expressed Equally

In studies of energy balance, a tradition has developed in which food intake is expressed per mouse (often showing no difference between the obese and the leaner mouse) whereas metabolic rate is shown per g body weight—with again the leaner mice showing an (apparently) much higher metabolic rate than the obese. The weight-reducing agent is thus said to increase thermogenesis that is uncompensated for by increased food intake and thus leads to weight loss. As will be understood from the above, such statements are incorrect. Clearly, when energy balance is discussed, the same units should be used for intake and expenditure (joules-in per mouse, joules-out per mouse).

3.1.6 Determination of Metabolic Capacity for Diet-Induced Thermogenesis

An implication of the (possible) existence of (adaptive) diet-induced thermogenesis is that the metabolic capacity should be increased. Provided that diet-induced thermogenesis is mediated by UCP1, the acquisition of diet-induced thermogenesis should be accompanied by an increased metabolic capacity. While such an increase can be estimated biochemically (see below), it is obviously

more compelling to see it manifest as a real increase in UCP1-mediated thermogenic capacity.

To the degree that diet-induced thermogenesis exists, the general concept is that it would acutely be induced by norepinephrine released from the sympathetic nerves in brown adipose tissue (activation through direct stimulation of the tissue by specific substances (e.g., food items or gut hormones) is also discussed but is not important for this discussion). It is therefore reasonable to suggest that the capacity achieved following an adaptive process that increases diet-induced thermogenesis can be estimated by injecting the mouse with a bolus of norepinephrine. This is also the case, provided that the experiment is performed with acutely fasted mice at thermoneutrality. However, such experiments have innate problems. One is that an injection of norepinephrine leads to an acute stress that lasts about an hour—which is the same time frame during which norepinephrine is effective. The injection has therefore to be given to anesthetized mice (but not under anesthesia induced by isoflurane or similar inhalation anesthetics since these inhibit brown adipose tissue thermogenic function [27, 28]; instead pentobarbital may be used). Another problem is that, in contrast to the tissue specificity provided by innervation, injected norepinephrine not only stimulates brown adipose tissue but also many other organs in the body, in fact any cell that possesses adrenergic receptors; thus, only an (undefined) fraction of the response can be ascribed to UCP1-mediated thermogenesis. Nonetheless, any increase in the thermogenic response to the norepinephrine injection in the treated versus the control mice can be considered a functional measure of the recruited capacity for diet-induced thermogenesis.

Instead of norepinephrine, injections of the β_3-adrenergic agonist CL316.243 can be performed. There are several advantages with this. One is that the mouse does not have to be anesthetized, because the response persists much longer than the injection stress response lasts. Another advantage is that CL316.243 has minimal effects on other tissues and thus the response can be interpreted as revealing UCP1-mediated thermogenesis. However, this selectivity can also be considered a disadvantage, since only a thermogenic response that involves β_3-responsive cells (i.e., adipocytes in brown and brite/beige adipose tissues) will be observed. A further disadvantage is that CL316.243 will not activate other adrenergic processes known to occur physiologically with norepinephrine, such as β_1-adrenergic processes and α_1-adrenergic processes, that stimulate or augment thermogenesis in brown adipocytes, and e.g. blood flow through the tissue is not induced via β_3-receptors. Thus, the total capacity for diet-induced thermogenesis may therefore be underestimated by only stimulating β_3-receptors.

3.1.7 Determination of Physiologically Relevant Metabolic Capacity

Although effects of adrenergic agents will reveal the total potential capacity of the thermogenic systems, it is only the thermogenesis that is actually evoked by the mouse that is physiologically relevant. This component is even more difficult to identify. It may be associated with a meal, and the increase in metabolic rate associated with a meal can be referred to as the thermal response to a meal. To the degree that this response is increased under conditions intended to stimulate diet-induced thermogenesis, it can be considered a demonstration of such thermogenesis. This is particularly the case if the increase is not seen when food is not offered to the mouse at normal eating times (e.g., [7]).

However, there is no inherent definition that diet-induced thermogenesis has to occur during meal time. It may be an ongoing process—but then we again approach the main problem in this area: the definition of diet-induced thermogenesis. Thus, chronically increased food intake would probably result in an increase in the masses of the liver and gut as well as an increase in physical activity due to the eating behavior as such. All of this would be visible as an increased metabolic rate. Is that diet-induced thermogenesis?

3.2 Tissue Characterization

If one adheres to the idea that diet-induced thermogenesis is caused by an adaptive process in brown and brite/beige adipose tissues, these tissues would be expected to show signs of recruitment. Here the main confounding issue is that diet-induced obesity will result in larger lipid droplets that, although metabolically inert, will visually dilute the metabolically active components. Thus, it will inevitably appear that, in obese mice, each parameter, when expressed directly or indirectly per volume, will be lower, whereas in leaner mice, these parameters will be higher. It thus appears that the tissue has become activated by the weight-reducing agent, and this apparent activation is then ascribed the functional role in the weight-loss process, as being the mediator of diet-induced thermogenesis. Often, however, changes in these parameters may be secondary to the reduction in the size of the fat droplets and have no functional significance, and a more integral view, considering the entire tissue, can demonstrate this.

Another confounding factor for interpretation is alterations in the tissue that can dilute functional thermogenesis-related enzymes (protein or mRNA) due to increases in other types of protein and mRNA, the so-called *pseudoatrophy* phenomenon [29]. Thus, when determined per aliquot/sample, the tissue may seem to possess less thermogenic capacity, but again, through an integral, organismal view, a qualitatively different conclusion will often be reached.

We will exemplify some of these possible pitfalls here.

3.2.1 Do Not Express Results per Square Centimeter

To follow the recruitment of brown adipose tissue, it is common to show (immuno)histochemical staining as sketched in Fig. 4. This figure could illustrate the density of blood vessels or nerves—or

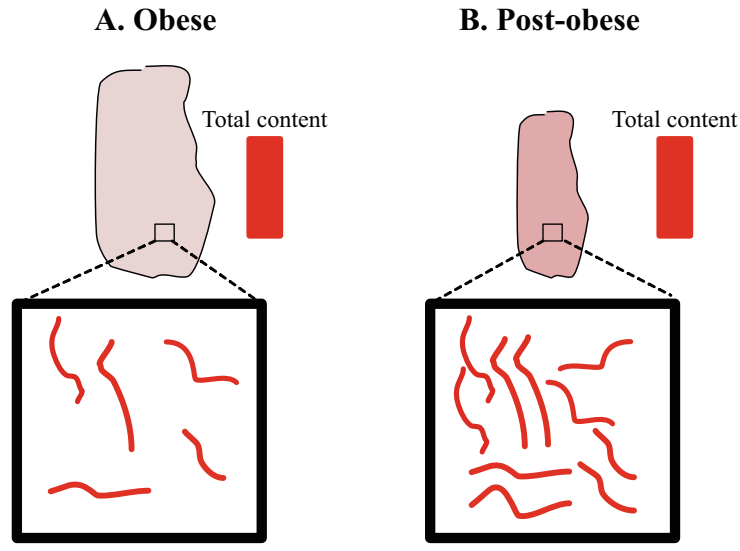

Fig. 4 The effect of obesity versus leanness on the appearance of adipose tissues. (**a**) In an obese state, the brown adipose tissue looks pale and each depot is rather large. When examined by, e.g., immunohistochemistry for, e.g., blood vessel density or nerve density (here illustrated by red lines), there is a large distance between these markers. In a post-obese state (**b**), the tissue looks darker brown—but is smaller. The immunohistochemical sections now show dense blood vessels/nervous supply. Thus, the tissue appears activated/recruited. However, if the total "amount" of blood vessels/nerves is estimated by measuring the total amount of the marker in the depot, it may not have changed at all (red bars). Thus, the change in appearance may not show anything other than the effect of the disappearance of lipid

even the result of standard H&E staining. As seen on the left (A), perhaps in a situation of diet-induced obesity, the tissue appears un-recruited, with large distances between the vessels/nerves/active tissue. After the administration of a weight-reducing agent, the picture in B is seen, with much denser vessels/nerves/active tissue. Thus, the conclusions from pictures such as these would often be that the tissue has become recruited, e.g., through growth of nerves or blood vessels, and this is a demonstration that diet-induced thermogenesis has been activated. However, the different densities may merely be a reflection of the amount of lipid in the tissue. With less lipid, the triglyceride droplets will shrink, and the cells will become smaller, yielding less distance between the vessels/nerves/cytoplasmic rims. In order to obtain a valid estimate of whether the tissue innervation/vascularization has indeed been increased, a possibility is to not only show immunohistochemical staining. In addition, the level of a relevant marker (e.g., tyrosine hydroxylase for innervation) should be measured in an immunoblot (western), yielding the level per mg protein. Further, the depot should be dissected out quantitatively, not just a sample, and from

that, the total amount of protein in the tissue can be determined. The total innervation can then be estimated as the total amount of tyrosine hydroxylase in the entire tissue, by multiplying the level per mg protein with the total amount of protein in the depot. As implied in Fig. 4, the result may well be that, despite the apparently convincing immunohistochemical staining showing "increased innervation", the total innervation may not have changed, i.e., there is then no evidence indicating augmentation of diet-induced thermogenesis.

Similarly, even if H&E staining appears to indicate that the protein content is higher in the tissue, total tissue protein content should be measured, to elucidate whether the same amount of protein has become denser (because of loss of lipid) or if a true functional growth of the tissue has taken place.

3.2.2 Do Not Express Results (Only) per Cubic Centimeter

With more sophisticated instrumentation it is possible to follow, e.g., nerves or vasculature in three-dimensional projections. The same reasoning applies here as for traditional immunohistochemical staining: the apparent changes in nerve density or vascular density should be accompanied by analysis of the relevant protein in the total tissue depot to be able to distinguish between dilution/concentration effects and genuine tissue recruitment.

3.2.3 Do Not Express Enzymatic Results per g Tissue Weight

The above considerations are evidently also valid for other types of indirect analysis of tissue thermogenic capacity, such as mitochondrial respiratory complex activities, etc. If again a comparison between lipid-filled and lipid-reduced conditions is made, a given enzymatic activity can be obtained per g wet weight of the tissue. However, if the mouse decreases in body weight and lipid content is reduced, the inevitable result is then necessarily that the density of everything else found in, e.g., a gram of the tissue, must increase, which may then be misleadingly reported as a sign of activated diet-induced thermogenesis. Again, only if analyzed at the total tissue level, can correct conclusions be drawn.

While this problem, i.e., expression per g wet weight, would seem to be circumvented by instead expressing enzymatic values per mg protein, this is not without problems as well, as discussed below (see 3.2.5).

3.2.4 Do Not Express Apparent thermogenesis per g Tissue Weight

In relation to studies of diet-induced thermogenesis, it is tempting to analyze thermogenesis of brown (or brite/beige) adipose tissue in a more direct way, as oxygen consumption. This may sometimes be performed by cutting the tissue into small pieces, placing these in an oxygen electrode chamber (e.g., an Oroboros) or in a Seahorse instrument, and merely measuring the resultant oxygen consumption (perhaps after adding a substrate such as succinate)—and, e.g., observing that the rate of oxygen consumption is higher in

preparations from the leaner mice, thus indicating that thermogenesis has become recruited. There are, however, several problems with this methodology and its interpretation.

One is that already alluded to above: when lipid amounts in the tissue are decreased, everything else, expressed per g wet weight, must necessarily be increased; the values for oxygen consumption per g tissue weight can thus be higher merely because there is more protein per g wet weight, without any recruitment having taken place.

A second problem is that the "state" that is measured is very undefined. In such preparations, it is not unlikely that what is measured is unregulated succinate oxidation occurring in damaged mitochondrial fragments in the tissue pieces. The information that can be obtained from this must necessarily be limited (although again, the values will presumably be higher in samples with less lipid).

A third problem arises when this type of preparation is stimulated, e.g., by addition of norepinephrine, to mimic nervous stimulation of the tissue. The problem that arises is a consequence of the inability of the (inner part of the) tissue pieces to receive sufficient oxygen. The thermogenesis/oxygen consumption observed will then only be a small fraction of that which is physiologically relevant. The problems with the oxygen supply in preparations of this type were classically followed by monitoring the level of NADH (versus NAD^+). If brown adipose tissue is stimulated with norepinephrine in situ (in the animal), the NADH level decreases, as would be expected, due to the induced uncoupling of the mitochondria [30]. This uncoupling will allow for electron flow from NADH to oxygen and NADH levels will thus decrease. However, when NADH levels are followed in tissue pieces from brown adipose tissue, the levels increase. This is because lipolysis is stimulated by norepinephrine, lipid catabolism is increased and more NADH is generated from, e.g., beta-oxidation. In the absence of sufficient oxygen in the tissue pieces, the electrons cannot flow to oxygen, and NADH accumulates. Thus, the tissue pieces become hypoxic when stimulated [30] and measurement of thermogenesis in this type of preparation is therefore not meaningful.

3.2.5 Do Not Express Results (Only) per mg Protein

A standard method to follow changes in tissues is to analyze tissue samples for, e.g., enzymatic activity and express this per mg protein. This is a natural way to work, e.g., if samples are taken from human muscle. However, in general, but particularly perhaps for brown and brite/beige adipose tissues, evaluation of the results is not necessarily straightforward.

A major problem is illustrated in Fig. 5. In Fig.5a, we have a situation where there is a given level of a component of interest (red) and some other (background) components (brown) present.

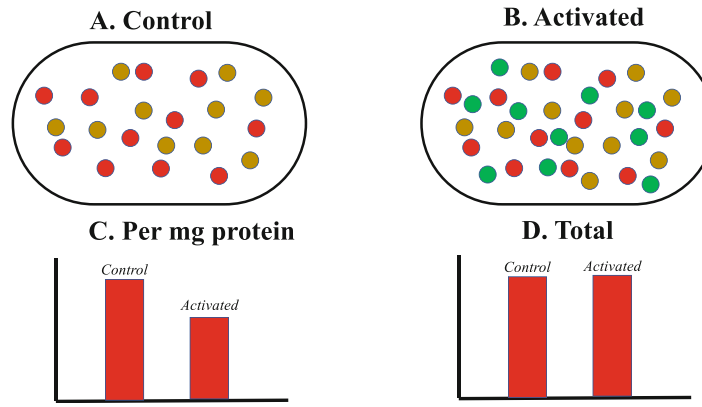

Fig. 5 The issue of pseudoatrophy. In (**a**), the control condition is shown. The red circles represent the protein-of-interest. There are also other proteins in the tissue (brown). A value is obtained for the protein-of-interest per mg protein (**c**, left bar). In (**b**), a change in the physiological conditions has meant that extra components (green circles) have been incorporated as constituents of the tissue (e.g., an increase in blood flow). In this situation, if the concentration of the protein-of-interest is calculated per mg protein, the result would be as that seen in (**c**) (right bar): a clear decrease (an atrophy). However, if the total amount of the protein-of-interest is measured, it is unchanged (**d**). Thus, the result in (**c**) is misleading and represents a state of pseudoatrophy. Similar outcomes may be encountered for mRNA levels

In Fig. 5b, a new physiological condition is observed that leads to an "invasion" into the tissue of new proteins. The relevant conditions could be several. One is what happens when the tissue goes from a dormant to an active state: blood flow increases markedly, and the tissue fills up with blood, containing red and white blood cells. There can also be a true invasion in the form of, e.g., macrophages, and changes in the hormonal environment can make the cells themselves start to produce, e.g., proteins for triglyceride synthesis in large amounts. In all these cases, there is not necessarily any change in the amount of the component of interest (red) but due to the dilution by the "invading" components, it would appear that the amount of the "red" component is decreased. This is what may be referred to as pseudoatrophy: that the data indicate a lowering of the component of interest—but in reality this has not taken place.

Again, the solution to the problem is to calculate the total amount of the component of interest in the tissue, to obtain physiologically relevant values.

3.2.6 To Express UCP1 Levels Only per mg Tissue Protein Can Be Misleading

Concerning diet-induced thermogenesis, the level of UCP1 protein attracts special attention, as it is generally considered to be the rate-limiting step for this thermogenesis. Its level can be determined by western blots, where it can be ensured that the band observed is of the correct molecular weight of ≈ 33000. However,

it is important that an antibody with high specificity is used since a large number of other mitochondrial carrier proteins have very similar molecular weights. Presently we can recommend Human/Mouse UCP1 Antibody MAB6158: R&D Systems as well as Human/Mouse Antibody Ab10983 from Abcam. These antibodies cannot be used uncritically in other tissues where they can cross-react with proteins of similar molecular weight (see also 3.2.14 for immunohistochemistry). In an extension of what was discussed above, it may especially be pointed out that "dilution-by-other-proteins" can clearly affect the interpretation of values for UCP1 when these values are only expressed per mg protein, i.e., the direct outcome in western blots. This issue is not only theoretical. Although not directly investigated for diet-induced thermogenesis, a confounding and apparent decrease in UCP1 (per mg protein) is observed in the very early phases of cold acclimation, probably due to the presence of more blood in the tissue [31]. Similarly, in *ob/ob* mice [32], and in glucocorticoid-treated mice [29], apparent decreases in UCP1 level are observed. In all these cases, it turns out that the total amount of UCP1 in the tissue is not decreased, nor is the magnitude of norepinephrine-induced thermogenesis. Thus, the decrease in UCP1 levels per mg protein leads to an erroneous conclusion concerning the thermogenic capacity of the tissue. Accordingly, if a weight-reducing agent should lead, e.g., to a decrease in anabolic (lipogenic) capacity of the tissue, this may be reflected in an increase in UCP1 level per mg protein (because anabolic enzymes are lost)—that may not reflect a true change in total UCP1 level and thus not indicate an augmentation of diet-induced thermogenesis.

3.2.7 UCP1 Protein Levels Should Be Expressed per Total Adipose Tissue Depot

If (changes in) thermogenic capacity are to be estimated from a biochemical measure, UCP1 is the natural candidate. It would generally be recognized that total muscle power cannot be adequately determined only by analyzing a single sample from muscle but must include an estimate of the total amount of muscle. Similarly, to obtain a total thermogenic estimate, the measurements should always include both UCP1 per mg protein and a measure of total protein in the depot, and only after multiplying these values can an estimate of total UCP1 and thus total thermogenic capacity be reached.

However, this could be said to be not fully adequate, because only the total amount (of UCP1) in one brown adipose tissue depot—normally the interscapular depot—is obtained. The implication will then be that similar changes occur in all brown adipose tissue depots. Optimally, all brown adipose tissue depots should be measured but this is seldom realistic. Estimates are that the UCP1 amount in the interscapular depot is about 1/2 of the total amount in brown adipose tissue [33] and that all depots change in a similar way.

Additionally, although there is good reason to think that thermogenesis and total UCP1 protein levels correlate positively, the relationship is not always proportional, i.e., an n-fold increase in total UCP1 would not always lead to an n-fold increase in total norepinephrine-induced, UCP1-mediated thermogenesis (whole animal oxygen consumption), for unknown reasons.

3.2.8 Use the Same (Arbitrary) Units to Express UCP1 Protein Amounts in Different Tissues

UCP1 protein is primarily found in the classical brown-fat depots, but may also be found in other adipose tissue depots in the body (referred to as brite/beige adipose tissue depots), mainly the inguinal adipose tissue. To obtain a biochemical value of the thermogenic capacity of the mouse, e.g., during diet-induced thermogenesis, the total amount of UCP1 in the inguinal depots should also be included. It is therefore important that the UCP1 values are comparable between the brown and the brite/beige depots. This necessitates that a common "standard" is present on all western blots (e.g., a sample of a homogenate of brown adipose tissue aliquoted and stored); this standard then enables all UCP1 to be expressed in the same "units." It is often necessary to dilute the standard when it is used for brite/beige tissues, as the UCP1 levels are generally much lower than in classical brown adipose tissue.

3.2.9 In Brown Adipose Tissue, it May Be Difficult to Identify a "Normalizing" Protein

For determination of UCP1 levels, we referred to it as being expressed per mg protein, when obtained from a western blot. Often for quantification of western blots, it is expected that the levels are expressed versus a protein referred to as a "loading control." Proteins such as β-actin, GAPDH, and vinculin are used. However, the high plasticity of brown adipocytes can make this procedure untenable. A perfect loading control would be a protein that does not change with physiological conditions, when expressed per mg protein. but there would then be no point in dividing by this value, as it would always be the same. Reports of apparent chnages in UCP1 that are the result of physiological changes in the loading control may be encountered. Thus, presently, expressing changes in specific proteins in brown adipose tissue is best done by expressing them per mg protein—and recalculating to the total amount in the depot. Ponceau or amido-black staining can indicate equal loading but even the pattern of these may change substantially in different physiological conditions due to the plasticity.

3.2.10 To Express UCP1 mRNA as a Proxy for UCP1 Protein (Thermogenic Capacity) Can Be Misleading

It is presently much more common to present data about proteins in terms of their mRNA levels rather than as protein amounts (or enzymatic activity). The main reason for this is that it is technically easier, particularly if a series of different proteins are examined. However, the apparent ease comes with problems of its own. Particularly, if UCP1 mRNA levels are used as a proxy for UCP1

protein levels, it must be remembered that UCP1 mRNA has a rapid turnover (hours) [34] and shows marked daily variation [35], whereas UCP1 protein has a much slower turnover (days) [36] and needs weeks to achieve new steady state levels. It is therefore not surprising that there may not be a direct correlation between UCP1 mRNA levels and UCP1 protein at a given time point, if this is during a transition state; these issues have been discussed in more detail elsewhere [37].

Due to the rapid induction of UCP1 gene expression by norepinephrine released from the sympathetic nerves in the tissue and to the rapid turnover of UCP1 mRNA, the levels of UCP1 mRNA an be seen as better proxies for acute sympathetic nerve activity in the tissue than for thermogenic capacity, particularly in physiological transition states.

3.2.11 Compare to the Same Standard Sample for mRNA Levels when Analyzing Different Tissues

qPCR techniques have an immense sensitivity span. Differences in expression of a million times can in principle be detected. A change of 10 Ct values corresponds to a difference of 1000 in gene expression level, a change from 35 to 25 is thus 1000-fold different, to 15 is 1,000,000-fold different, and Ct values below this may then approach at least 10,000,000-fold difference in expression. UCP1 mRNA levels that give Ct values of 20 and below are needed to produce UCP1 protein in sufficient amounts for it to induce uncoupling in isolated mitochondria. It is thus difficult to ascribe physiological significance to gene expression levels 1000 times lower (i.e., Ct values around 30). Such low UCP1 mRNA can be detected in certain tissues under certain conditions. However, it is important to express the UCP1 mRNA data in such a way that the absolute values are evident. This is often not the case, as UCP1 mRNA levels in different tissues are often expressed normalized to the "starting value" in that tissue. Thus, a substance suggested to induce diet-induced thermogenesis may increase the UCP1 mRNA level from 10to 100 molecules in a brite/beige adipose tissue depot; this will be reported as a ten-fold increase. At the same time, it may increase the UCP1 mRNA level in a brown fat depot from 1000 to 1500 molecules; this will be reported as a 50% increase. Whereas it is evident that 500 molecules can be expected to produce more heat than 90 molecules, the result may confusingly be presented as a dramatic increase in brite/beige adipose tissue thermogenic capacity (1000%), with an almost insignificant increase in brown adipose tissue thermogenic capacity (50%).

Thus, in diet-induced thermogenesis studies, for thermogenic relevance, UCP1 mRNA levels should be expressed with the same units in all the different depots.

3.2.12 Beware of the "Divisor" Normalization

Traditionally, in order to check for possible differences in cDNA synthesis efficiency , gene expression values for a gene-of-interest are normalized to a "housekeeping" gene. Whereas the epithet "housekeeping" implies that all cells express these genes (for general cell function), the term does not necessarily imply that the levels of these genes are the same in all cells, nor that they are constant under all conditions. Studies of gene expression are therefore often accompanied by attempts to identify a good housekeeping gene to normalize to. In reality, this amounts to identifying a gene that does not change between conditions—but if such a gene is found, it is used to divide all data by the same constant (—that should then yield a result that is identical to that obtained without normalization) (cf. 3.2.7).

Particularly for brown and brite/beige adipose tissues, the plasticity of the tissues makes it difficult to identify "good" normalizing genes. A further problem that may be encountered in this search is to look only at the Ct values and conclude that they are practically identical (e.g., "they are all 22–23"). However, due to the logarithmic nature of the Ct values, such a difference represents a doubling of the divisor level. If there is a doubling of expression of the normalization gene, this will necessarily result in an apparent halving of the expression of the gene-of-interest, even if no such change has actually occurred.

We therefore think that it would be preferable to report gene expression in "absolute" values, i.e., as 2^{-Ct}. This means in reality that the levels are expressed per mg RNA.

3.2.13 Total UCP1 mRNA Levels per Tissue Depot Are Physiologically Meaningful

As discussed above for protein levels, an organismal point of view should be attempted also for mRNA levels. In reality, within the limitations that half-lives etc. constitute, mRNA levels are used as indicators of enzymatic activity (providing information on UCP1 thermogenic capacity), since protein amounts are generally determined by mRNA amounts. Although this relationship is not always valid (and examples where it is not can be found even in brown adipose tissue contexts [38]), this must still be the basic idea in steady states. The normal representation of UCP1 mRNA levels is then per mg RNA (or per normalizing gene). Rather, for an organismal view, the total amount of UCP1 mRNA is obtained by multiplying UCP1 mRNA levels per mg RNA with the total amount of RNA in the tissue (the total amount obtained in the isolation procedure). To obtain this value, it is therefore important also in this context to quantitatively dissect the total depot.

Due to the large differences in RNA levels between different tissues, this adjustment may alter the thermogenic significance that should be given to UCP1 gene expression in different tissues. Notably, the total amount of RNA is, e.g., much lower in epididymal adipose tissue than in brown adipose tissue, despite the fact that the tissue is much larger [39]. Thus, presenting the data in this way yields a result that is physiologically more meaningful.

3.2.14 To Determine UCP1 Recruitment by Immunohistochemistry Is Difficult

One way to infer thermogenic adipose tissue recruitment in studies of diet-induced thermogenesis is to show immunohistochemical staining of the tissue with anti-UCP1 antibodies. There are several problems in the interpretation of such data.

One problem is that many commercial UCP1 antibodies are not sufficiently specific. The monoclonal antibody we recommend for western blots cannot be used, as it is made in mice, and secondary antibodies to this antibody therefore react with all mouse antibodies in the tissue slices. The specificity problem can best be verified by using tissue from UCP1 KO mice as a negative control, or at least tissue pieces from a non-UCP1 expressing tissue with high mitochondrial content (e.g. kidney).

Further problems arise not least because the tissues change their composition during recruitment. Firstly, there is the cm^2 effect discussed above (Fig. 4): even if there is no true change in total amount of UCP1, it will appear so, because a larger fraction of the section will be non-lipid. Secondly, stimulation of the tissue may increase the number of mitochondria without increasing the amount of UCP1. Although many antibodies are made against short UCP1 peptides, there is a risk that they recognize other members of the mitochondrial carrier protein superfamily. This is particularly the case because when used for immunohistochemical staining the antibodies are often added in high concentrations, increasing the risk for unspecific reactions. There is no simply solution to this; again analysis of tissues from a UCP1 KO mouse should increase the validity of the observations.

3.2.15 **An increase in UCP1** may be secondary to rather than causing slimming Although an increase in the amount of UCP1 would normally be considered evidence for an evoked diet-induced thermogenesis, the possibility unfortunately exists that observed increases in UCP1 gene expression are secondary to the weight loss, rather than being the cause of it. It would seem that e.g. in inguinal adipose tissue a population of adipocytes inherently exists where the UCP1 gene is already open for transcription when the cells are adrenergically stimulated. During an effective weight loss ("browning") treatment, the adipose tissue depots are necessarily adrenergically stimulated, as the stored triglycerides are degraded in the process. This means that UCP1 gene expression is necessarily stimulated in these responsive cells; the increase in UCP1 is thus not causative of the weight loss through activated thermogenesis but is caused by the weight loss. Due to the low initial level of UCP1 gene expression normally observed in the inguinal adipose tissue, the thermogenic capacity of the induced UCP1 is negligible, although the relative increase in UCP1 gene expression can be marked. Why such a cell population exists in the inguinal adipose tissue is not clarified. Again, the only simple way to examine whether UCP1 is causative of or secondary to the weight loss that is induced by a "browning" agent is to perform the examination in UCP1 KO mice, with the complications that this in its turn implies.

3.3 Blood Glucose Estimation

Experiments related to diet-induced thermogenesis/obesity often also relate to blood glucose homeostasis (as diet-induced obesity is often associated with prediabetic and diabetic states). There are methodological issues relating to the obesity, even in these measurements.

3.3.1 For Glucose Tolerance Tests, Do Not Inject Glucose in Proportion to Body Weight

A standard experiment to demonstrate altered glucose homeostasis in obese mice versus slim mice is the glucose tolerance test. In these experiments, a single dose of glucose is injected into the mouse (often intraperitoneally). This leads to a rapid increase in blood glucose levels, and the trajectory of glucose normalization is a measure of the diabetic state (Fig. 6). However, the injections generally given are 2 g per kg body weight. This means that a substantially greater amount of glucose is injected into an obese versus a lean mouse, often nearly twice as much. However, as the aqueous volume in which the glucose is dissolved in the body is essentially the same in the two cases, the curve for glucose disposal will necessarily be very different (Fig. 6). Thus, at first sight, there appears to have been a very convincing improvement: when the mouse is obese, it is also (pre)diabetic. When it becomes leaner, the (pre)diabetes disappears. In reality, this is the inevitable outcome if 2 g glucose per kg body weight is injected (as it is in practically all published cases). This can be presented as a positive effect of BAT activation on improving glucose tolerance (if the obesity is considered to be reduced due to BAT activity). However, because of the procedure, this difference is either largely exaggerated or does not exist.

This does not mean that an obesity-induced reduction of glucose tolerance does not exist, rather that methodologically either the same dose of glucose (e.g., 40 mg per mouse) should be used

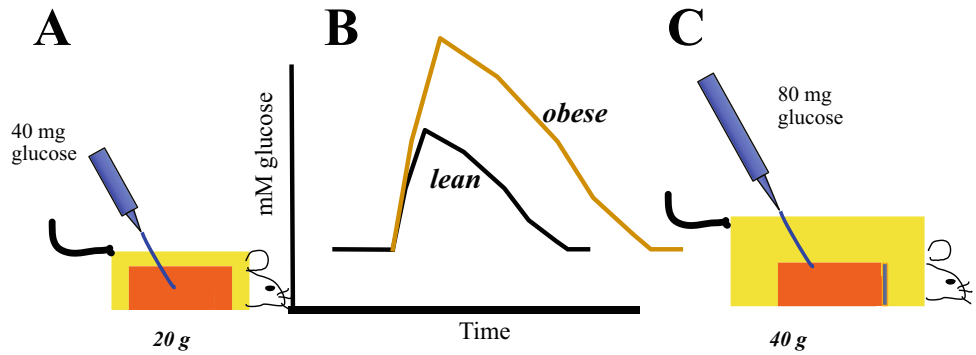

Fig. 6 The inherent effect of obesity on the outcome of glucose tolerance tests. In the standard procedure, a dose of glucose proportional to body weight is injected into the mouse (**a**). This results in a glucose tolerance test, principally as sketched in the lower curve in (**b**). If the same body weight-paradigm for glucose dose is used for obese mice, a higher amount of glucose is injected, but it will be dissolved in (practically) the same amount of lean mass (i.e., mainly water) (**c**). This will thus, everything else being equal, result in the top curve in (**b**). Note that this will necessarily occur even without any true change in glucose tolerance

both in lean (i.e., normal) mice and in obese mice—or, somewhat better, that the same dose per g lean mass should be used. This allows for the possibility to discern the true effect of obesity on the glucose tolerance test. These issues have been discussed in several papers [40, 41].

3.3.2 For Euglycemic, Hyperinsulinemic Clamps, Do Not Express the Results per g Body Weight

A much more labor-intensive but basically also more informative procedure is the euglycemic, hyperinsulinemic clamp, where a high (saturating) dose of insulin is continuously injected into the mouse and the amount of infused glucose needed to compensate for the glucose disposal is measured.

However, routinely, also here the result (that will initially be expressed as moles of glucose per animal per min) will be expressed divided by body weight. Again, inevitably, the obese mouse will present with an apparently decreased glucose disposal capacity, whereas the lean mouse will appear to have a greater capacity. Thus, also in these experiments, the result should be presented without division, i.e., as moles of glucose per animal per minute.

4 Final Remarks

It is very likely that the phenomenon of diet-induced thermogenesis does exist and that it is located mainly or entirely in BAT and is due to UCP1 recruitment and activation. With time, it may indeed prove possible to recruit diet-induced thermogenesis to ameliorate obesity. However, it is of paramount importance for confidence in studies that underlie such undertakings that the measurements, results, and interpretations that constitute progress in the field avoid deceptive pitfalls. We hope that the compilations of possible pitfalls above will facilitate development of a deeper physiological understanding in this important field. If not, translational studies will have a high risk of failing, as they would be based on unfounded conclusions from preclinical studies.

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