Doctoral thesis in Physiology

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Thermal physiology and metabolism

Interplay between heat generation and energy homeostasis

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Abstract

Mammal metabolism is intimately connected to the maintenance of body temperature. While metabolic pathways invariably produce heat as a by-product, the natural heat present in the environment also plays a role in defining the adaptive metabolism and general physiology of an organism. This thesis aims to discuss basic aspects of energy expenditure and their interactions with energy stores and body composition.

In Paper I, we apply a new technique – high-resolution laser-Doppler imaging – to describe physiological regulatory features of adrenergically-stimulated blood flow in brown adipose tissue, and evaluate the validity of blood flow as a parameter to estimate nonshivering thermogenesis.

Paper II focuses on the central regulation of body temperature. In the absence of bombesin receptor subtype-3, mice present an altered neurological body temperature setpoint, while peripheral thermogenic capacity remains intact. We conclude that brown adipose tissue malfunction is not the cause of the hypothermia observed in this mouse model.

Paper III incorporates measurements of body temperature to the energy expenditure of different sources: basal metabolic rate, physical activity, thermic effect of food, and cold-induced thermogenesis. We describe basic aspects of dynamic insulation, energetic costs of circadian variation and hypothesize that physical activity may change the body temperature setpoint.

Paper IV describes methodological issues related to glucose tolerance tests in obese mice. We conclude that the erroneous scaling of doses may affect the interpretation of metabolic health in mouse models, and suggest a new methodology.

Paper V describes the outcomes caused by the expression of the human Cidea protein in adipose tissue of mice and suggests that this protein may clarify the link between adipose tissue expansion and healthy obesity.

Paper VI explores the dissociation between thiazolidinedione-induced adipose tissue "browning" and reduced blood glycaemia. We demonstrate that although this pharmacological class tends to induce some level of brown adipose tissue recruitment, this phenomenon does not define its antidiabetic effects.

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Natural science does not simply describe and explain nature;
it is part of the interplay between nature and ourselves.

Werner Heisenberg

To the warmth of life

Enclosed with this thesis are the following papers:

I. **Adrenergically-stimulated blood flow in brown adipose tissue is not dependent on thermogenesis.**
Abreu-Vieira G, Hagberg CE, Spalding KL, Cannon B and Nedergaard J.
In press - Am J Physiol Endocrinol Metab, doi: 10.1152/ajpendo.00494.2014 (2015)

II. **Regulation of body temperature and brown adipose tissue thermogenesis by bombesin receptor subtype-3.**
Lateef DM, **Abreu-Vieira G**, Xiao C, Reitman ML.
Am J Physiol Endocrinol Metab. 306:E681-7 (2014)

III. **Integration of body temperature into the analysis of energy expenditure in the mouse.**
Abreu-Vieira G, Xiao C, Gavrilova O, Reitman ML.
In press - Molecular Metabolism, doi:10.1016/j.molmet.2015.03.001 (2015)

IV. **On adequate procedures for glucose tolerance tests in obese animals.**
Abreu-Vieira G, Bengtsson T, Petrovic N and Nedergaard J.
Manuscript submitted

V. **Cidea improves metabolic profile through expansion of adipose tissue.**
Abreu-Vieira G[1], Fischer AW[1], Mattsson C, de Jong JMA, Shabalina IG, Rydén M, Laurencikiene J, Arner P, Cannon B, Nedergaard J, Petrovic N.
Manuscript under review

VI. **Novel thiazolidinediones distinguish between (UCP1-independent) antidiabetic effects (MSDC-0602) and adipogenic and browning-inducing effects (MSDC-0160) of classical thiazolidinediones (rosiglitazone).**
Abreu-Vieira G, Kalinovich A, Cannon B, Nedergaard J.
Manuscript in preparation

[1] Authors contributed equally to this work.

Contents

Abbreviations

BAT	Brown Adipose Tissue
BMR	Basal Metabolic Rate
BRS-3	Bombesin Receptor Subtype-3
CIDEA	Cell death-inducing DFFA-like effector A
CIT	Cold-Induced Thermogenesis
C_{max}	Peak Concentration
C_o	Initial Concentration
dT_b	Defended Body Temperature
EE	Energy Expenditure
F	Bioavailability
GTT	Glucose Tolerance Test
HR.LDI	High-Resolution Laser-Doppler Imaging
K_e	Elimination Constant
LCT	Lower Critical Temperature
LPS	Lipopolysaccharide
MET	Metabolic Equivalent of Task
PA	Physical Activity
PAEE	Physical Activity Energy Expenditure
POA	Preoptic Area
RMR	Resting Metabolic Rate
$T_{1/2}$	Half-life
T_a	Ambient Temperature
T_b	Body Temperature
TEE	Total Energy Expenditure
UCP1	Uncoupling Protein 1
V_d	Apparent Volume of Distribution

1 Preface

It is with great pleasure that I write this PhD thesis. Over the last 4 years, my focus has been to investigate the interactions between the fields of thermogenesis, obesity and bioenergetics. The focal point of interest was attempting to understand and to quantify the different ways by which experimental mouse models utilize energy. Because of our laboratory's tradition, the study of thermogenesis – the catabolism of substrates causing heat to be released – is a major topic. While all projects started in order to understand the function of brown adipose tissue, methodological challenges have given origin to seemingly unconnected spin-off projects. Those, in turn, became what I consider to be important steps towards describing general terms of metabolism, including brown adipose tissue basic physiology.

Instead of presenting an extensive review of published data based on pre-established concepts, this thesis is structured as a discussion of the concepts per se. In this context, physiological hypotheses may sound deceptively straight-forward, but should be taken as a nuanced and personal collection of thoughts on the nature of the observed phenomena.

Due to this reflexive character, few literary references are used in some sections, while logics is used in an attempt to craft sound rationales and to inquire into their implications. At times, compound nouns that have been previously abbreviated may appear once again in its extended form. This deviation from standard scientific writing is a decision aiming to increase the clarity of the text, mostly where several specific terms are used sequentially.

All concepts discussed are, at the time of writing, expected to be applicable to future investigations of human metabolism. While the attempt may be overoptimistic, the fun of trying is certainly worth the effort.

2 The concept of heat applied to physiology

The field of thermal physiology aims to investigate the biological implications of temperature to the function of living organisms. This includes adaptations in face of environmental challenges, as well as the metabolic demands causes by these adaptations. But before introducing the physiological studies, it is necessary to define the terms to be used, and this includes the notion of temperature. There are different ways to conceptualize it, ranging from random atomic agitation in a molecule, to complex vibrational patterns termed phonons. Here, however, we apply the primitive notion of hotness, referred to as heat. Experimental results are always indicated according to the Celsius scale, and the costs of heat generation are defined by kilocalories. Although other units like watts or joules may be more adequate to physical experimentation, the ultimate focus on metabolism guides the decision to apply the caloric concept to the data. No additional physical nuances are necessary to define temperature at the present level of biological investigation. It is, though, important to recall the ways by which temperature varies in a body and the associated physiological relevance.

Heat production may happen as a by-product of metabolic processes, i.e., as the inefficient part of molecular transformations that causes part of the energy not to be used for work, but instead, lost as heat. It may also be produced by organized thermogenesis when an endothermic organism struggles in face of a colder-than-desirable ambient. Heat transfer, in its turn, occurs naturally by different modalities. Conduction is the direct transfer of heat between two substances in contact with each other. This classification includes the warmth leaving the bare feet in contact with the ground, or dissipating through an organ when generated by a focal point (e.g. a mitochondrion).

If the heat is being carried from one point to another by a moving substance, the mode is called convection. Convective currents are perhaps the most important for the temperature balance of an organism. Heat produced during thermogenic processes are readily spread throughout the body by the blood circulation. This is the reason why, e.g., the brain can be kept warm by heat generated in distant organs. Convection also plays a fundamental role in heat loss: air currents surrounding an organism will

facilitate the thermal dissipation to the environment (considering it is at a lower temperature than the organism), therefore the great benefit of nest building and shelter-seeking behaviors. Radiation is the transfer of thermal energy in the form of light waves, as exemplified by the heat we receive from the sun. All bodies, including mammals, emit heat by radiation. During the winter, some small mammals will bury themselves in the snow. This decreases heat loss by radiation, once the light/heat emitted is reflected by the snow back on the animal. Because of the low body temperature of the animal, the radiative heat loss light falls outside the visible spectrum for humans. When warmed up enough, as in the case of heated metals, the radiative glow becomes visible. It can be speculated that our lack of capacity to see low-temperature radiation may have evolutionary purposes: bright colors caused by high temperatures becomes suddenly visible to indicate the danger of flames, magma, or the plasma of lightning during a storm.

Life is strictly associated to ambient temperatures. Every organism works optimally in a range of environments, and climate alterations force species through natural selection (i.e. death of many individuals and survival of the species in case pre-existent random mutations turn useful for the new environment). It is interesting to consider the reasons why body temperature is strictly controlled in mammals. It is not trivial to rationalize the reason why homeothermy might have evolutionary advantages over poikilothermy. The first classification is applied to animals that try to keep their body temperatures within a rather stable range, as opposed to poikilotherms. Now assume that, for whatever reason, an organism evolved in the direction of homeothermy. Its organs and cellular functions will likely be more adapted to an optimal range of temperatures, while large environmental fluctuations could turn disastrous. In this homeothermic scenario, the capacity to perform thermogenesis (endothermy) is greatly advantageous in relation to ectothermy (i.e. when organisms rely on heat absorbed from the environment in order to maintain their own temperature constant). Because no common endothermic ancestor for, e.g., mammals and birds seems to have existed (Fong et al., 2012), it is likely that the "warm-blooded" feature is a product of convergent evolution. Convergence, in turn, is a common trait of environmental pressure stimulating nonrelated species to adopt similar beneficial strategies that will ensure survival. The choice of keeping a stable temperature, allied to the capacity to endogenously produce heat, is thought to have allowed animals to spread over wide

geographical locations, to endure cold winters, and to perform metabolic conversions at optimal temperatures (Grigg et al., 2004, Portner, 2004, Kurbel, 2014). Convergent evolution is then a reason supporting the existence of evolutionary advantage for endothermic organisms.

In summary, heat exchange with the environment, along with endogenous control of thermogenesis, are fundamental in defining the metabolism and the life of an organism like ourselves. Several functions seem to have coevolved to support temperature homeostasis: energy storages for later combustion (white adipose tissue), integration and processing of information regarding temperatures (nervous system), and direct production of heat (brown adipose tissue and muscles). As it happens to be, the organs performing these functions are also intimately related to the modern human diseases of obesity and diabetes. Therefore, there is a necessity to investigate physiological functions connected to temperature control, as well as their interplay with caloric balance. Just like heat generation has defined the life history on our planet, the understanding of thermogenesis (and the capacity to manipulate it) may turn greatly beneficial to the future health of our own species.

3 Compartments of heat generation

When attempting to describe metabolism, different terms are currently used to classify energy expenditure and its integrated parts. Each individual choice of nomenclature tends to be based on the immediate focus of the study being performed, as well as on the methods available for data acquisition and analysis. In order to aid the search for therapies against metabolic alterations, it is fundamental to establish sound rationales for the concepts to be employed when evaluating metabolism. Based on this necessity, this section (based on papers II and III) attempts to craft distinctions between the elements of energy expenditure and to discuss the conceptual implications and practical applicability of the parameters.

3.1 Thermic effect of food

Thermic effect of food (TEF) is generally defined as the energy necessary for the digestion, absorption, transport and storage of macronutrients. The values of TEF are approximately 10% of the energy content of the ingested food, and can be thought of as an energy "tax" on food assimilation. Therefore, in a situation when an organism keeps its body composition constant (i.e. when food intake equals caloric expenditure), around one tenth of the measured metabolic rate will be expected to be due to TEF.

The percent energy (over its own energy content) necessary for each macronutrient to be metabolized is assumed to be approximately 2.5% for lipids, 7.5% for carbohydrates, and 25% for proteins (Trumbo et al., 2002). These values are, however, assumed based on empirical experimentation. Any attempt to precisely describe the TEF of a specific molecule would necessarily have to incorporate complex stoichiometric calculations, as well as a plethora of metabolic pathways that are seldom in steady state. It would then be honest to assume that discussion on the precise values of TEF seem to be ahead of our time and methodological capabilities.

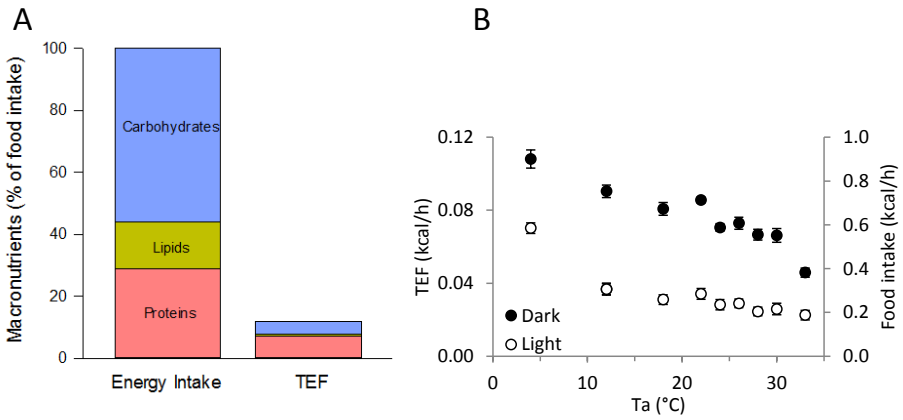


Figure 1. Thermic effect of the food. **(A)** Proportion of macronutrients composing the chow diet used for papers II and III, along with each macronutrient's contribution to the thermic effect of food (TEF). **(B)** Representation of food intake and TEF variability over a range of ambient temperatures (data from paper III).

It is important not to confuse TEF with a parallel phenomenon: diet-induced thermogenesis (DIT). The conceptualization of DIT is that organs (e.g. brown adipose tissue) may have an adaptive response to macronutrients (Rothwell and Stock, 1979). This response could be modulated by factors within the organism, producing a different thermic response to the same amount of ingested macronutrient, although it is described to happen primarily during caloric overfeeding (Rothwell et al., 1982, Himms-Hagen, 1984); therefore the conceptual dissociation made between DIT and TEF. A careful reader will realize that these definitions do not preclude controversy: in theory, every process included in the TEF could be somewhat modulated, implying that the lines between TEF and DIT are thinner than would be desired when attempting to measure their contributions to heat generation. For the sake of simplicity, it could be suggested that the term TEF would be better used when the contribution of food-related energy consumption is pondered against that of distinct nature (e.g. from physical activity or induced by cold exposure). On the other hand, DIT may be better suited to the analysis of how a tissue or an organism can be conditioned to elicit different thermic responses to similar nutritional stimuli.

In our measurements (Paper III), TEF was quantified by directly converting the food intake to the consensus values of TEF, proportionally to each macronutrient's

presence in the food. This is demonstrated in Figure 1B, where data points, representing the results obtained from a group of mice fed a standard chow diet, can simultaneously demonstrate TEF (primary Y axis) or food intake (secondary Y axis). The beauty of this analysis resides in its simplicity: in this case, 12% of the energy intake is utilized for its own metabolism. Additionally, colder temperatures stimulate food intake, equally increasing the heat produced as TEF. We considered TEF to be independent of additional variables in order to make it easier to quantify the other elements of energy expenditure and heat generation.

3.2 Physical Activity

Physical activity (PA) incorporates every kind of skeletal muscle contraction that may or may not generate a visible movement of an organism or segment of the body. For a contraction to occur, energy has to be transferred from different metabolic pathways. Regardless of the substrate utilized as fuel, the costs of PA can be assessed by analyzing O_2 consumption (unless anaerobic glycolysis is taking place), CO_2 production, and/or heat released from the body. It is very intuitive to assume that a significant part of the energy transferred from the catabolism of chemical substrates to the mechanical contraction of muscles is released as heat. We humans possess effective mechanisms to dissipate the heat that is generated during exercise when the weather is hot; the most pronounced being sweating and skin vasodilation. The heat generated by PA can be assessed in experimental animal models, as exemplified by a scatter plot correlating the assumed body temperature trend in the face of varied levels of PA (Figure 2). In this representation, data collected during light or dark phases of the day have been divided due to circadian variation of body temperature (which will be addressed later in the setpoint-related sections of this thesis).

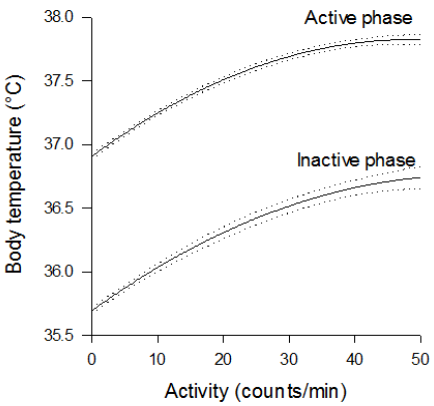


Figure 2. Correlation between PA and T_b. (Data from wild type mice used in Paper II).

The conceptualization of physical activity is rather trivial, but its measurement is not. Physical activity energy expenditure (PAEE) arises from every muscle being contracted in the body. Muscles work as chains of segments that constantly redistribute their forces of contraction, and likely the associated metabolic costs (Myers, 2001). However, some muscle activities do not produce limb movement but have significant physiological effects (i.e. isometric contractions (Mitchell and Wildenthal, 1974)). Additionally, anatomical variations of the muscle insertion in the bones could theoretically cause biomechanical alterations, modifying the metabolic cost per unit contraction force (e.g. kcal/N) (Brinckmann et al., 2002). For the cited reasons, the measurement of PA is still a technical issue in the quantification of energy metabolism. Therefore, when measuring PA, data acquisition has to be clearly explained, and the methodological limitations made explicit.

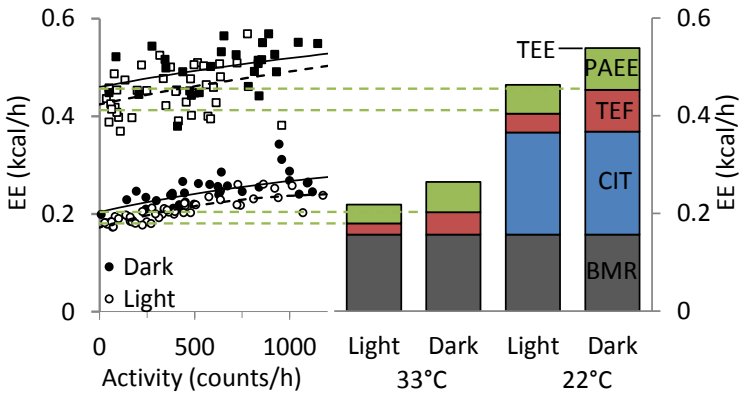


Figure 3. Energy expenditure related to physical activity. Scatter plot relating activity and total energy expenditure. The Y intercept of the trendline represents the mathematical approximation of a mouse's caloric costs at rest. The delta between TEE and Y intercept represents the PAEE. Additional energy expenditure related to other compartments are also represented. PAEE, physical activity energy expenditure; TEF, thermic effect of food; CIT, cold-induced thermogenesis; BMR, basal metabolic rate. Data referent to Paper III.

In Paper III, PA was quantified indirectly from a kinematic perspective: mice were positioned in cages that transmit an animal's spatial information to a receptor; changes in position were considered as movement caused by PA. However, although being an indirect method, the analysis of the generated data has yielded very interest-

ing results. As demonstrated in Figure 2, PA can be positively associated with an increased body temperature, and the temporal gap between PA and increased core temperature of the mice was found to be approximately 4 min (Lateef et al., 2014). In Paper II, a correlation demonstrating the effects of PA on body temperature allows for a clearer definition of the anapyrexic phenotype of BRS-3 knockout mice, which is most easily detectable during low levels of activity.

A further analysis of the energetic costs of PA can be made by measuring a mouse's O_2 consumption and CO_2 release in an indirect calorimeter, and associating this data with the values of activity (Figure 3). This way, the value where the activity regression line reaches its Y intercept can be assumed to be the total energy expenditure minus the PAEE (Resting metabolic rate). Additionally, when the values of PAEE are divided by PA, one can obtain the values for the specific caloric cost per unit activity (as presented in Paper III).

3.3 Cold-induced thermogenesis

Cold exposure is a hazard to homeothermic animals. It is important to notice, though, that cold does not exist as a physical entity. Instead, it is perceived by an organism as the feeling of heat loss from its body to an environment that happens to be at a lower temperature. Whenever a homeothermic endotherm does not generate enough basal heat to sustain its T_b, some extra amount has to be generated. The energy expenditure related to this heat production is therefore termed cold-induced thermogenesis (CIT).

Brown adipose tissue (BAT) has as its primary function the maintenance of body temperature through the combustion of metabolic substrates. This happens because of its unique capacity to uncouple thermogenesis from the synthesis of ATP, thus creating a highly inefficient mechanism by which heat is released without macroscopic mechanical work being performed (Nedergaard et al., 2001). In BAT, uncoupling protein 1 (UCP1) has recently been proposed to exist as a functional helical tetramer anchored in the inner mitochondrial membrane (Hoang et al., 2013). When bound by free fatty acids (which reach high intracellular concentrations during lipolysis), the nucleotide inhibition of UCP1 and proton transport across the mitochondrial membrane can occur (Cannon and Nedergaard, 2004). When the proton gradient is

dissipated, heat is produced (as indicated e.g. by increased T_b in Figure 4C (of note, thermogenesis does not necessarily increase T_b since heat can in some situations be dissipated)). To conceptualize this form of thermogenesis, one could think about chemical potential energy (the gradient) causing protons to be moved at high speed through the membrane. This mechanical force causes molecular agitation, perhaps because of UCP1 molecular vibration or by attrition/collision of protons with atoms on the other side of the membrane. Because one definition of temperature is the state of molecular agitation in a system, this kinetic agitation in this scenario represents heat itself.

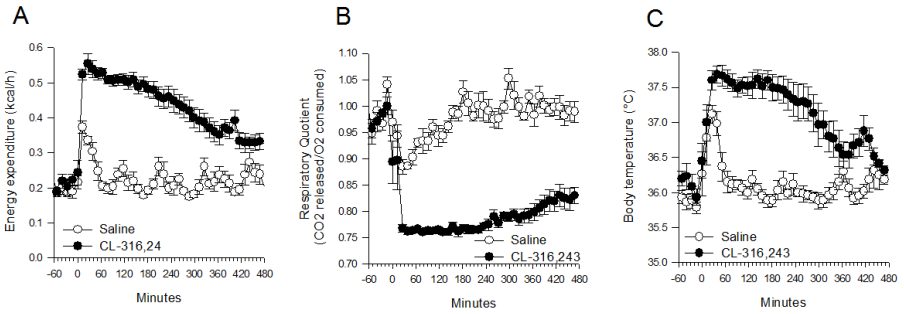


Figure 4. Stimulation of nonshivering thermogenesis. Cold-induced thermogenesis was simulated by injecting CL-316,243 (β3-adrenoceptor agonist) in ambulatory wild type mice at 30°C. **(A)** Energy expenditure increases as brown adipose tissue becomes activated. This is accompanied by **(B)** decreased respiratory quotient (indicating increased net lipolysis) and **(C)** increased body temperature. Data originally from Paper II.

For lipolysis to occur, causing fatty acids to trigger UCP1-dependent thermogenesis in BAT, the tissue undergoes adrenergic activation by the sympathetic nervous system (Hardman and Hull, 1970). Norepinephrine present in nerve endings (Cottle and Cottle, 1970) is released and binds to several receptors, the most prominent being β1- and β3-adrenoceptors (Bengtsson et al., 2000). Interestingly, β3 receptors may also be used to activate thermogenesis in human BAT (Cypess et al., 2015).

It is interesting to ponder on the reason why mammals have evolved to produce heat preferably from BAT. While muscle shivering is known to be fully capable to warm up the body in the absence of UCP1 (Golozoubova et al., 2001), it seems to have deleterious long-term effects, causing mortality rates to be higher over prolonged periods of time (Golozoubova et al., 2001). The reason for this is not very clear, but

it is possibly related to intracellular muscle damage (Aydin et al., 2008). It can be imagined that brown adipocytes that evolved to generate heat will possess additional lines of defense against heat shock damage and oxidative stress caused by their own chemical reactions. It may also be that muscle tissue has not had the same pressure to cope with constant shivering, as organisms usually have to endure only temporary cold stresses. Additionally, it is natural that shivering temporarily decreases the effectivity of the muscle and alters coordination. In the wild, proper motor control is not only good for an animal, but fundamental to define life and evolution. It is unlikely that an animal caught during substantial shivering will have the same fighting capacity to avoid predation or to swiftly respond to environmental danger. On the other hand, BAT thermogenesis is not easily perceptible, and is not known to overlap with or compromise any other function of the body. Therefore, this could be considered an evolutionary advantage of nonshivering over shivering thermogenesis.

3.4 Basal metabolic rate

Also known as basal energy expenditure (Trumbo et al., 2002), the common understanding of this cost is that it represents the energy necessary to "keep the system working". Most likely, it is associated with the costs for the enzymatic machinery to convert substrates, as well as to energy necessary for the turnover of proteins and organelles. In any case, the basal metabolic rate (BMR) can be thought of as the energy required for an organism's homeostasis at its lowest level of necessity: at rest, in a post-absorptive state, at thermoneutrality and euthermic. This definition ultimately excludes the thermic effect of ingested food, cold-induced thermogenesis, as well as costs related to physical activity. In Paper III, we added an extra requirement for the measurement of BMR: that the animal shall be in its "inactive" phase of the day (i.e. light phase for mice). This was made to ensure the validity of the idea of "lowest energy necessary", since circadian alterations in body temperature could affect the quantification of BMR. The energy expenditure caused by pregnancy, lactation and growth are also not included in this definition, although it is possible to bend the rigidity of this rule and discourse on how, e.g., pregnancy would affect the BMR (Lof et al., 2005, Forsum and Lof, 2007).

4 Central Regulation of Body temperature

4.1 Body temperature setpoint

Endothermic animals tend to maintain their body temperatures within a strictly regulated range (exemplified in Figure 5A). The conceptualization of a body temperature setpoint is old and has been much debated, mostly from a biological perspective. Interestingly, the validation of energy expenditure data can make use of the setpoint concept from a thermodynamic perspective, and increase understanding of heat balance mechanisms.

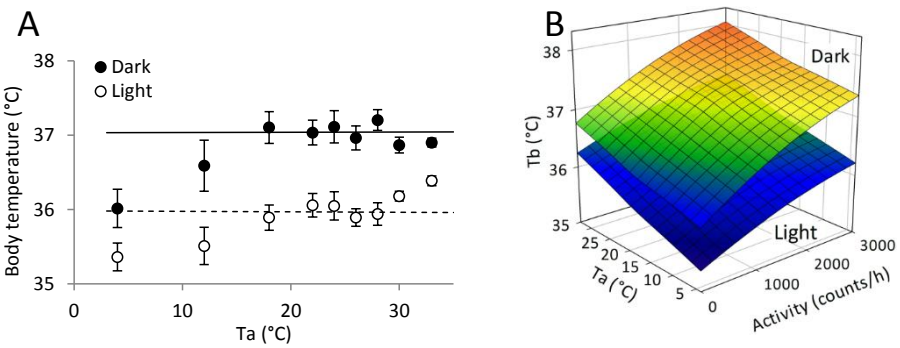


Figure 5. Physiological oscillation of body temperature.

Firstly, it is important to define the real body temperature (T_b) values measured from the animal. In our case, telemetry devices implanted in the intraperitoneal cavity transmitted real time data on the core Tb while mice were analyzed inside indirect calorimeters. Those values were compiled to define the average Tb during dark or light phases of the day over a range of ambient temperatures. The values were close to 37 °C during the dark and around 36 °C during light phase. Of note, mice are nocturnal animals, being awake and active mostly during the dark phase. As ambient temperature (T_a) decreases, mice tended to present lower Tb, and presumably equally decreased energy expenditure (EE). Of note, although in my writings I consider this decrease to be a regulated form of hypothermia, most scientists would disagree with this interpretation and consider this phenomenon to be caused by a failure in performing thermogenesis at higher levels of demand. Nevertheless, points used for further

analysis of EE were those between collected at the stable range of 18-28 °C (Paper III), which is methodologically acceptable according to both interpretations.

If the simultaneously measured values of EE are plotted against the T_a where they were measured, a line can be drawn and extrapolated to the X intercept. This value (when X = 0) is conceptualized to represent the defended body temperature (dT_b), according to the following reasoning: in order to maintain T_b stable, the organism needs to produce heat (by burning substrates present in the food or internal reserves) at the same rate as heat is lost from the body to the environment (Scholander et al., 1950a). If the T_a decreases but T_b is kept stable, metabolic costs should increase in near perfect linearity, according to the principles described by Fourier's law of heat conductance. Therefore, heat exchange between the body and the environment is supposed to be proportional to the temperature gradient at these points. Accordingly, the point where heat generation (a surrogate for heat loss under the described conditions) is zero (X intercept) indicates the temperature where organism and environment are in thermal equilibrium, that is, the dT_b.

This elegant method has been used for over half a century for the analysis of several biological parameters, such as the rate of heat loss in an organism and the definition of the thermoneutral zone of an endotherm (Scholander et al., 1950a). Nevertheless, even though the theory behind the method is perfectly sound, its use has been shown to be far from banal. The analysis of total energy expenditure hardly extrapolates into dT_b values that match the measured T_b, and there have been controversies in the question of whether different compartments of heat production (e.g. TEF, CIT and PAEE) would substitute each other, undermining attempts to calculate individual contributions (Virtue et al., 2012). For these reasons, our analysis was based on validating the energy expenditure compartments data and their extrapolated dT_b by using the actual T_b values as compass. According to this, the closest approximation is achieved by excluding the PAEE from total energy expenditure lines. This decreased the analysis error by 0.5 °C and 3.5 °C on data acquired during light or dark phases, respectively. It is uncertain, though, if values from TEF should be included or excluded from the analysis, since it is difficult to find a biological rationale for it. Anyhow, our analysis seems to create a paradox: the best extrapolated dT_b is found when PAEE is subtracted from total energy expenditure, but T_b (used as a reference for the "correct" dT_b) is measured concomitantly with total energy expenditure. This apparent

violation of thermodynamics implies that our application of Newtonian physics to living organisms is somehow flawed. Possible explanations for this error may lie in 1) the principles that insulation (the concept given to the mechanisms limiting heat loss) is maximally recruited below thermoneutrality, or that dT_b represents a single value "desired" by the central nervous system. If dT_b were to be a stable value to be kept, then the extra heat generated under some level of cold exposure would be easily dissipated to maintain a stable T_b. At the same time, other sources of heat generation (included in the concept of cold-induced thermogenesis) would be easily shut down, neutralizing the costs of activity below thermoneutrality (as previously proposed (Virtue et al., 2012)).

4.1.1 Setpoint shift: fever

Perhaps the most straight-forward example to describe an elevation in the body temperature set-point is fever, a well-known event for all of us. It is characterized by increased T_b, the feeling of cold, and it is usually associated with an infectious state. Fever is not, though, simply represented by a body that happens to be at a higher-than-average temperature (i.e. hyperthermia). Its definition requires the organism to "want" to be warmer, thus characterizing a shifted central setpoint.

4.1.2 Setpoint shift: physical activity

In contrast to the hypothesis that exercise would be "free" in cold, PA seems to have its own costs, implying that a new T_b is aimed at during activity (Figure 6). This claim is equivalent to proposing that T_b increases in a fever-like fashion during exercise. To estimate its magnitude, hard evidence is necessary and will be provided as follows: if the setpoint, or dT_b, is altered during activity, then this effect (increased T_b) should be seen regardless of the environmental temperature. This happens to be the case, as demonstrated in Figure 5B, where the measured activity positively correlated to the T_b over a wide T_a range, including those temperatures below the thermoneutral zone. Data pointing at the same direction were provided in Figure 2, when mice analyzed at 21 °C (well below thermoneutrality) increased T_b with exercise.

Once more, if the increased T_b was only a side effect of activity, and the heat generated simply a by-product of muscle contraction, then there would be no reason for PA's effects to be seen in cold. From a metabolic perspective, heat generation in cold is expensive and the energy would not be wasted to increase T_b over a stable setpoint. The biological implication of this proposed paradigm shift is that, when searching for the anatomical origin for exercise-related metabolism (i.e. PAEE), we may have to look further than muscle, possibly into organs that possess a high thermogenic potential such as liver and brown adipose tissue.

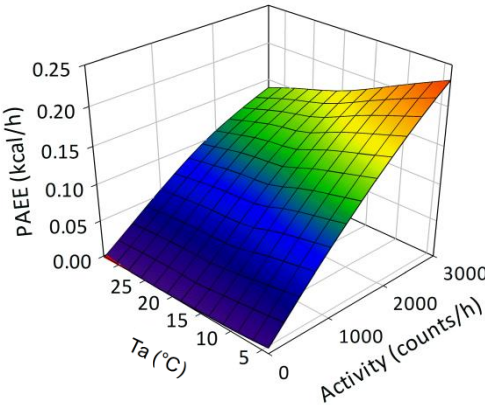


Figure 6. Variation of the energetic costs of physical activity in relation to ambient temperature. Caloric expenditure related to a single amount of ambulatory activity is higher in cold. The lower basal T_b of mice in cold demands a higher energy expenditure for the physical activity T_b setpoint to be reached (data from Paper III).

It is interesting to note that the theory of increased T_b setpoint during activity does not necessarily conflict with the concept of thermoregulatory heat substitution, postulated to occur at various levels in different species below thermoneutrality (Humphries and Careau, 2011). In fact, the shape of our curves suggests that a plateau is reached at high levels of PA. When activity-related changes in energy expenditure (Figure 3) or T_b (Figure 2) are plotted, the best fitting trendlines are not linear, but polynomial. This is very likely due to the heat substitution occurring at high intensity exercise (high PA over time), when (most probably) CIT mechanisms would gradually shut down once the new T_b setpoint is achieved. In this case, it could be postulated that heat substitutions starts occurring when the core T_b is around 1 °C higher than the resting dT_b, the latter varying according to circadian rhythm. The evolutionary advantage for the higher temperature seems to lie in biomechanical improvements of muscle function (James, 2013).

4.1.3 Setpoint shift: circadian variation

Following the discussion on setpoint-driven changes in T_b, we arrive at an expected aspect of T_b fluctuation: circadian variation. Much has been argued about the daily variations in the T_b of mammals, including whether it is centrally regulated (e.g. via hypothalamic pathways) or simply an effect of the metabolic heat production related to activity and food intake. Circadian variation of T_b seems to be caused by a central setpoint shift. Were it not to be the case, then below thermoneutrality, there should be no increased T_b or higher energy expenditure during the active phase (in the case of mice, the dark phase, as demonstrated in Figure 7).

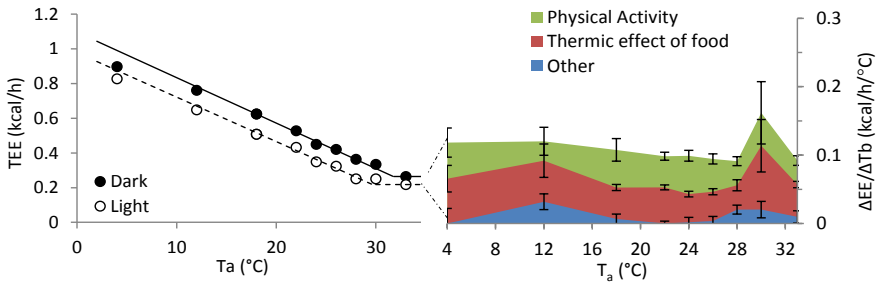


Figure 7. Energy expenditure related to circadian variation of body temperature. The circadian variation in Tb is associated with variations in activity and thermic effect of the food. This proportion is kept stable across the entire spectrum of analyzed ambient temperatures, supporting the concept of a coordinated increase in the T_b setpoint.

In paper III, we have measured the specific costs of the compartments of energy expenditure during light and dark, as well as the variation in T_b. This way, it became possible to define the sources of the heat produced (Figure 7). At most ambient temperatures, both physical activity and thermic effect of the food seem to contribute similarly to the higher "active phase" T_b, while there is a possibility for other sources to contribute, presumably cold-induced thermogenesis. The variations in energy expenditure (dark-light Δ) are proportional to the ΔT_b, making the cost of keeping the body warmer during the active phase to look amazingly stable over the full range of tested ambient temperatures (the cost is approximately 0.1 kcal/h/°C). It is important to note that the Q_{10} effect is most likely not involved in the altered metabolic rate cause by changes in temperature setpoint (Heldmaier and Ruf, 1992).

The reason behind the circadian variation of T_b is not clear, but its well-regulated features suggest that there may a functional significance for an organism's physiology. Future studies may be able to define the optimal temperature of events happening at the cellular level, as well as their significance to functions associated with every specific part of the circadian cycle.

4.2 Neural pathways

The nervous system functions as the most precise and effective way to integrate several environmental stimuli in order to swiftly trigger a systemic response. The thermoregulatory system connects different parts of an organism aiming at maintaining temperature homeostasis, according to a centrally-regulated setpoint. It is no secret that "setpoint" is simply a concept used by those investigating the thermal control of endotherms. It arises from the observation that body temperature is kept under seemingly strict regulation, with some level of oscillations around a constant value. The most interesting fact is that oscillations do not happen at random, but instead seem to follow meaningful cycles and situations, as previously described in the sections on fever, physical activity and circadian variation. If one aimed to define an anatomical location for the setpoint, it would have to be an area with enough neuronal input to sense the external ambient temperature, to be sensitive to chemical signals from the body (e.g. toxin-triggered signals during infection), and significant neural output to trigger systemic responses. The hypothalamus, more specifically the preoptic area (POA), qualifies for all these requirements: it receives cutaneous signals of external temperature, which are transmitted through the spinal cord and the lateral parabrachial nucleus, until it arrives to the POA (Nakamura and Morrison, 2011). It also possesses prostaglandin E2 receptors (EP_2) (Hosoi et al., 1997, Zhang and Rivest, 1999), which are involved in the perception of inflammation/infection, and can be triggered[2] by the systemic presence of e.g. bacterial lipopolysaccharides (LPS) (Matsuoka et al., 2003). Once all incoming signals are processed, the resulting neuronal output is given as signals that descend to the dorsomedial hypothalamus and subsequently toward the periphery of the body, causing acute changes in insulation (e.g. vasoconstriction and

[2] EP_2 receptors are in fact activated by prostaglandins, which are found in higher blood concentration when the organism senses the presence of bacterial wall fragments (i.e. LPS).

piloerection) and heat generation (from muscles and BAT). The thermogenic neuronal pathway is similar for muscle shivering and UCP1 activation in BAT (Nakamura and Morrison, 2011). It is known that animals tend to shiver only when BAT does not suffice to maintain the body warm. This concept can be applied to the late-autumn shivering of humans, which disappears throughout the winter as BAT becomes optimally recruited, as well as for UCP1 KO mice, that will shiver indefinitely in the cold.

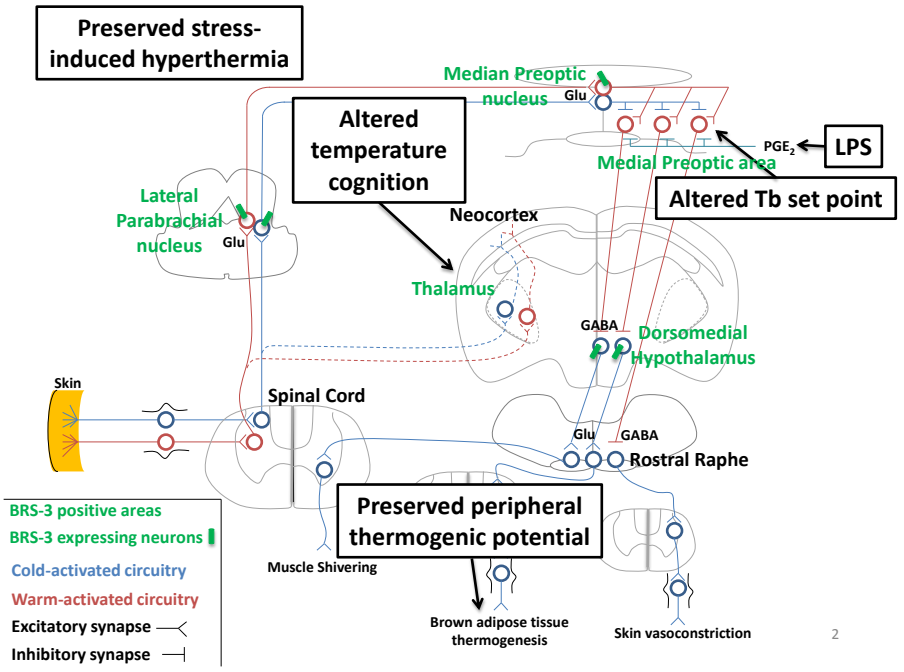


Figure 8. BRS-3 presence in the central thermoregulatory pathways based on Nakamura's thermoregulatory pathways model (Nakamura and Morrison, 2011), [3H]Bag-2 binding in mouse brain (Guan et al., 2010) and co-localization of BRS-3 expression with neurotransmitters (Zhang et al., 2013). Highlighted boxes indicate the functional findings on BRS-3 KO mice and their association to anatomic locations (Paper II).

It is, therefore, likely that the neuronal distinction between activating one or another form of heat generation may lie in the signal threshold. Following this line of thought, low-intensity signals would easily trigger BAT, but be insensitive for muscles. In the absence of BAT, more signals would pass the "muscle threshold", triggering shivering thermogenesis.

In Paper II, we described the functional alterations of mice lacking the bombesin receptor subtype-3 (BRS-3). The background of this study is that BRS-3 has been shown to influence metabolic functions related to diabetes (Matsumoto et al., 2003, Nakamichi et al., 2004, Feng et al., 2011), while mice lacking this receptor develop long-term mild obesity and have been described to have lowered metabolic rates (Ohki-Hamazaki et al., 1997, Maekawa et al., 2004). Because BRS-3 can influence body temperature (Guan et al., 2010, Guan et al., 2011), we set out to define whether brown adipose tissue plays a role on BRS-3 regulation of body temperature. As highlighted in Figure 8, we found that although BRS-3 KO mice have altered temperature recognition (i.e. chose a lower ambient temperature when given the choice), and maintain a constantly decreased body temperature, these changes are most likely caused by an altered neurologic setpoint, since brown adipose tissue thermogenesis was found to be intact when directly tested.

A final consideration of thermoregulatory pathways: as presented in Figure 8 (Nakamura's model), signals ascend from the thalamus towards the neocortex, where the ambient temperature can be consciously understood by the animal. The absence of known connections between the thalamus and other thermoregulatory centers (i.e. the dorsomedial hypothalamus) gives rise to interesting implications. Assuming that the model is correct, then thermoregulatory behaviors are not only controlled by the cortical recognition of temperatures zones, but at a more instinctive "subconscious" level. In this scenario, although the organism may have understood what temperature would be more adequate for its present needs, other defense mechanisms may be connected to a deeper sense of well-being related to the thermal zone. It would then be implied that those additional mechanisms may be somewhat related to the limbic system, which is in fact anatomically closer to the thermoregulatory centers than to the cortex. Of course, this reasoning lies within the realm of speculations, but the thought of unexplored mechanisms is too tempting ignore.

5 Maximum thermogenic capacity

In our results, cold induced a decrease in Tb (Figure 5 of this thesis, data from Paper III). However, the hypothermic effect of severe cold is not to be taken for granted. A given Ta may lead to reduced Tb or cause no alteration at all. Therefore the need for some unifying explanation of the data. A possible underlying reason could be that Ta is not the single factor defining heat loss in an organism: the convective air currents, ground conduction, and lost heat being reflected on the surrounding surfaces play a fundamental role in defining how "cold" it actually is.

But instead of presenting a long explanation of the modalities of heat transfer affecting the measurements, the important concept to work on at this point is the biological reason why hypothermia occurs in some well-fed animals but not in others, while all are kept in e.g. 4°C.

As previously cited, heat loss is highly variable at a given Ta, but stable in a determined setting (i.e. experimental hardware setup). As heat loss and heat generation are, below thermoneutrality, theoretically the same value[3], a likely biological rationale for the apparent hypothermia is that heat generation reaches its peak (the maximum thermogenic capacity of an organism) depending on the experimental design and associated heat loss. Thus, it can be hypothesized that, e.g., doubling the air flow that passes through the mouse being measured in an indirect calorimeter, would increase the metabolic demand and heat generation according to the doubled convective heat loss. If the decreased T_b results from the maximum thermogenic capacity having been reached, then there should be some kind of unifying variable to be applied to the data acquired in different settings.

As a starting point, one could assume some proportionality between basal metabolic rate and maximum thermogenic capacity (Bennett and Ruben, 1979). This is not a trivial assumption in view of BAT function: it is known that UCP1 does not possess constitutive activity (i.e. protons do not leak across the membrane solely due

[3] Heat generation and loss are seen as the same value based on the premise that body temperature is kept stable, but this principle should not be extrapolated ad libitum.

to UCP1's presence), so there is no proportionality between BAT thermogenesis-related metabolism and what is measured in the same tissue at rest. On the other hand, proportionality becomes clearer once additional organs are incorporated to compose a greater metabolic portrait of the organism (summarized by (Hayes and Garland, 1995)).

For conceptualization purposes, let us now focus on the cardiopulmonary and hepatic functions. In order to maintain the high catabolic rates necessary for a day in cold, nutrient intake and processing (gastrointestinal and hepatic functions (Kvist and Lindstrom, 2000)), as well as gas exchange and diffusion (cardiopulmonary function[4]), have to be significantly increased. This adaptation includes altered physical dimensions and higher basal organ function. As a fact, cold-acclimated animals adapt visceral organs, have higher metabolic rate, and are expected to have a higher total thermogenic capacity (Nespolo et al., 2001). This way, a concept that naturally develops is that an organism will be able to increase its heat generation to (approximately) a certain fold over its resting metabolism. Therefore, this hypothesis utilizes the concept of metabolic equivalents of a task (MET) (Jette et al., 1990). If the resting or basal metabolism is defined as a reference value (equal to 1 MET) then thermogenesis can be observed as the task equivalent to a certain fold of the metabolic reference, be it the resting metabolic rate (RMR), BMR, or other. The main difference between these terms is that BMR does not incorporate the thermic effect of food, while RMR does. Assuming that thermogenesis will be accompanied by increased food consumption (and therefore TEF), it can be argued that not much precision will be lost when opting for the use of RMR in place of BMR.

Overall, this hypothesis predicts that **1)** mice will be capable of achieving a maximum sustained thermogenic capacity of, e.g. 4 METS. This given value is used without evidence, obviously demanding further research for its actual definition.

2) Whenever a mouse is forced over the maximum sustainable thermogenesis limit, hypothermia will be evoked as a means to conserve energy.

[4] According to Fick's principle, the maximum thermogenic capacity (Vo_2 max) of a mammal equals its cardiac output (Q) multiplied by the arteriovenous difference in O_2. Hypothesizing that an active BAT depletes the circulating O_2 levels (as it mostly likely does to its immediate environment), then cardiac function can become a major limiting factor for thermogenesis.

3) In cold-acclimated mice, baseline reference levels of energy expenditure (this could include several modalities of adaptations) will be increased, allowing for an increased maximum thermogenic capacity. If the principle of proportionality turns out to be correct, the alterations in energy expenditure (both basal and maximum) will not affect the MET value. It is possible, though, that it is necessary to introduce some scaling power to be able to apply this principle to an intraspecies comparison.

4) Heat exchange with the environment (at measurement) will change the temperature where hypothermia is evoked.

The recognition that the calorimeter setup affects the rate of heat loss, hereby exemplified by the alteration in body temperature at 4°C, gives rise to significant implications. Since the same concept is valid for measurements of metabolic rates at 21°C (the standard room temperature), as well as for its comparison to thermoneutrality, it seems rather pointless to argue deeply about the numerical effects of environmental temperature on metabolic rate. That is, based on a series of experiments, it could be argued that housing mice at room temperature will trigger enough cold stress to increase food intake and O_2 consumption by 50%, by 2 fold, or by 2.5 fold. While the values are of extreme relevance for the interpretation of metabolic data, such discussion is at risk of eventually becoming misleading: in principle, this fold variation is caused by the surrounding physical environment (i.e. the heat loss caused by the experimental setup), as much as by the measured ambient temperature.

6 Heat distribution and insulation

6.1 Blood flow

Perhaps the most important feature allowing multicellular organisms to maintain high levels of metabolism, the circulatory system is crucial for nutrient and gas diffusion, communication between organs (the endocrine system) and for temperature regulation. Heat produced in an organ (e.g. BAT) warms up the blood and is carried by convection to other organs, where it is released based on the thermal gradient. At the same time, heat loss can be avoided by skin vasoconstriction in distal regions of the body, as is the case for birds' legs, mammals' tails, or human limbs exposed to cold. This creates a shelling effect that can be measured as a temperature gradient: extremities are kept colder than vital organs in the thorax and abdomen. On the other hand, skin vasodilation is evoked to allow heat to be released from the organism, as exemplified by the scarlet-colored cheeks of light-skinned runners, at the end of a high-intensity exercise bout. Once the heat is transferred to the body surface, in humans, sweating is triggered to increase evaporative heat loss at higher ambient temperatures (Colin and Houdas, 1965).

In brown adipose tissue, blood flow is locally increased when a larger supply of oxygen and nutrients is needed (Heim and Hull, 1966, Kuroshima et al., 1967, Foster et al., 1980). Additionally, thermogenesis seems to correlate with higher BAT blood flow (Foster and Frydman, 1978). This increase depends on the sympathetic drive received by BAT (Engel et al., 1992), as well as on the production of the vasodilator nitric oxide (Nagashima et al., 1994, Nisoli et al., 1997, Sotornik et al., 2012).

Past investigations on BAT blood flow have used diverse methods such as radiolabeled indicators (Bullard and Funkhouser, 1962, Jansky and Hart, 1968), invasive cannulation of veins (Heim and Hull, 1966), labeled microspheres (Foster and Frydman, 1978, Ma et al., 1986), laser-Doppler flowmetry (Engel et al., 1992), injectable fluorophores (Nakayama et al., 2003), PET-scan (Muzik et al., 2013) and ultrasound (Baron et al., 2012). All cited techniques present some specific drawback: some require terminal procedures, others demand the use of contrast-enhancing agents

(sometimes meaning radioactivity). Therefore, we established the use of high-resolution laser-Doppler imaging, a noninvasive technique that allows for longitudinal, in vivo analysis of subcutaneous adipose tissue blood flow in mice (Paper I).

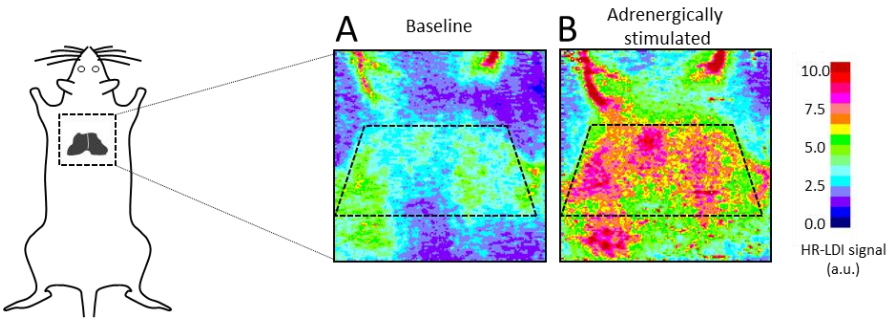


Figure 9. Measurement of brown adipose tissue blood flow. Imaging of the dorsal region of a mouse, containing the classic interscapular brown adipose tissue deposit. Tissue blood flow was visualized **(A)** in the basal state and **(B)** post norepinephrine injection. Blood flow was assessed by high-resolution laser-Doppler imaging. The establishment and validation of the method are presented in Paper I.

The biological rationale for the increased blood flow in active BAT is clear: high metabolic rates demands a sufficient supply of oxygen and also macromolecules to be used as energetic substrates. Thus, BAT activation is directly associated with higher blood flow. While the concept is correct, a common misconception that easily follows this reasoning is that thermogenesis itself can trigger an increase in tissue perfusion. In Paper I, we describe that UCP1-KO mice, albeit incapable of performing thermogenesis, maintain wild-type blood flow regulation in BAT. This implies that the use of blood flow data as the sole parameter to detect BAT activation is flawed and possibly misleading.

6.2 Insulation

There are many ways one can refer to heat loss and its associated physiology. While “heat loss” per se is the heat transfer from the body to a colder environment, and its unit is understood as “conductance”, the term “insulation” aggregates biological functions that modulate the rate of heat loss. Normally, birds and mammals defend a body temperature higher than the surrounding environment (Ruben, 1995). When

this difference is small enough so that basal metabolic rate and insulation suffice to stabilize body temperature close to the defended setpoint, this range of ambient temperatures is considered to be the thermoneutral zone for that specific organism (Scholander et al., 1950a). When insulation reaches its maximal point (i.e. the organism uses all available strategies to minimize heat loss), cold-induced thermogenesis is triggered to maintain temperature balance.

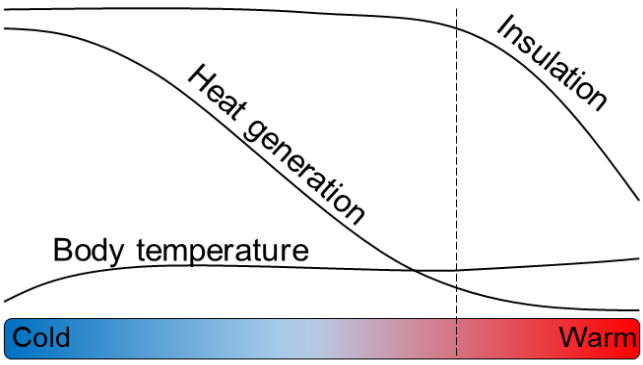


Figure 10. Relationship between heat generation and heat loss. Body temperature is maintained by an intricate balance between heat generation (thermogenesis) and modulation of heat loss (insulation). Dashed line represents the lower critical temperature (LCT). Above the LCT, body temperature is kept stable by the use of dynamic modulation of insulation, until the upper critical temperature is reached and body temperature will invariably increase.

The biological components of insulation can be thought of as being passive or dynamic. The passive class is composed of the rather stable physical insulation, as the thick layer of fat in seals, or the fur coat of an arctic animal. Even though we are now terming these features "passive", they are by no means immutable. The seal fat can obviously change over time, based on the caloric balance of the animal, and the insulating characteristics of fur can be changed by several fold when submerged in water (Scholander et al., 1950b). These slight variations are, however, still very stable when compared to, e.g. the dynamic changes mammals can perform in cutaneous vascular tonus, when attempting to modulate the rates of heat loss (Stewart, 1913, Ingram and Legge, 1971, Bornmyr et al., 2001). Other than being an abstract physiological parameter, insulation forms a fundamental triad together with thermogenesis and body temperature (Figure10) (Scholander et al., 1950a). Defective dynamic insulation in

mice (in this case, decreased capacity to perform tail vasoconstriction, leading to increased heat loss) has recently been shown to cause an increased demand for brown adipose tissue thermogenesis (Warner et al., 2013, Warner and Mittag, 2014).

In Paper III, we estimated the insulation of mice in the range 4 to 33 °C. The dynamic insulating capacity, calculated as the amount of heat loss (therefore termed conductance), can be estimated by dividing the energy expenditure by the temperature gradient between body and environment. Since energy expenditure is "corrected" per temperature, this method allows for a more precise estimation of the lower limit of thermoneutrality, as well as for the adaptive changes in dynamic insulation occurring while energy expenditure is stable (i.e. within the thermoneutral zone). Additionally, the heat loss after death could be calculated by a combination of measurements of post-mortem body temperature decay rate and the heat accumulated by fat and lean masses. According to these measurements (and comparison with in vivo rates of heat loss), dead animals lose heat at a rate approximately 5-fold higher than when alive. Since the physical insulation was not altered after death, these data serve as an exemplification of how efficient mammals are in conserving body temperature by dynamic means (further discussion in Paper III).

7 Body composition and energy homeostasis

7.1 Scaling caloric costs

Over time, scientists have attempted to find unifying mathematical factors to explain and calculate the energy necessary to supply a determined amount of life. Past scaling approaches include the association of energy expenditure with cardiac function (Bishop and Spivey, 2013), nutrient distribution (West et al., 1997), body-brain size (Yu et al., 2014), and most classically, with factors based on a direct conversion from body mass[5] (Kleiber, 1947, Blackmore, 1969, Parizkova et al., 1984, Donhoffer, 1986, Refinetti, 1989).

It is still unclear whether a scaling factor based on Kleiber's law (¾) would be ideal to normalize energy expenditure, or if exponentials closer to the surface-volume ratio[6] (⅔) would be more appropriate. There has been significant research done on these factors, with occasional argumentation being sparked by the proponents of each factor (White and Seymour, 2003, Packard and Birchard, 2008).

There have also been attempts to unify different variables defining the scaling of energy expenditure (White and Kearney, 2013), or even to explain deviation in factors based on trivial issues such as the analyzed sample size (White and Seymour, 2005). A general similarity connecting the studies is an explanation of energy expenditure based on unifying mathematical factors that can be extrapolated over a range of species that span over several orders of magnitude (in body mass). On the other hand, our work has attempted to analyze the energy expenditure of mice at a smaller scale of variability. With this intent, different components of heat generation (or heat loss) have been correlated to different parts of body composition: lean and fat mass of each mouse (Paper III). This dualism is of course not a rule of nature, but a human definition that serves the purpose of distinguishing between the metabolically active

[5] The list of references presented here is very modest and serves the sole purpose to guide future reading. An attempt to summarize all studies that have been performed on scaling would take the length of a full thesis, and even then some questions would very likely remain.

[6] The two-thirds-power scaling is based on Euclidean geometry, where e.g. a sphere will vary its surface by two thirds of its volume change. Its application to mammals refers to the concept of heat loss to the environment (surface) as being determinant of energy expenditure.

lean mass, and the inert lipids that compose the fat mass. The following sections present the stronger associations encountered in our analysis: fat mass and heat loss, as well as lean mass and basal metabolic rate.

7.1.1 Heat conductance and fat mass

In paper III (Figure 11A of this thesis), we describe the strong positive correlation between fat mass and conductance, while lean weight does not seem to affect the rate of heat loss. These data are slightly counterintuitive, since fatness is commonly associated with a capacity to insulate the body against heat loss (i.e. there should have been a negative correlation). Therefore, some discussion on the possible causes of this phenomenon is necessary. One possible scenario is to assume fat weight to be simply the body composition parameter that best reflects body weight variation within this group of animals. Body weight, in turn, is related to the body size of the organism. In this straight-forward reasoning, the association between fat weight and increased conductance is caused by the increased surface area.

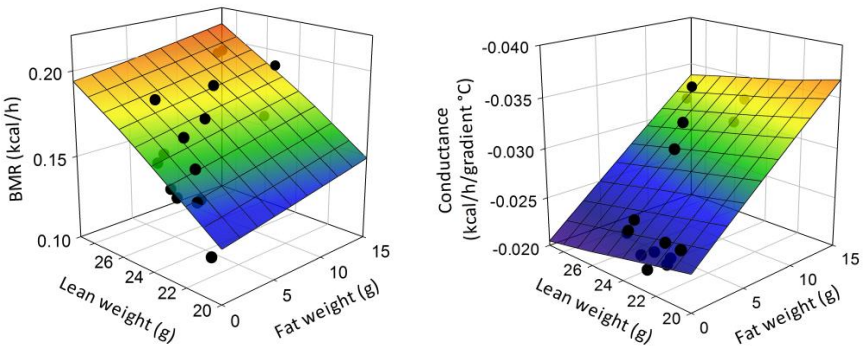


Figure 11. Correlation between body composition and thermal balance. (A) Positive correlation between basal metabolic rate and lean weight. **(B)** Association between fat mass and conductance (rate of heat loss) of mice. Data presented in Paper III.

Interestingly, while one could wonder whether a fat mouse would be more protected against cold, the present paradox is that fatness will also increase surface area and heat loss. That is, the counterintuitive result is that fat animals happen to have less insulation than lean, and require extra heat to be generated at similar levels of cold exposure. This heat produced falls within the classification of cold-induced thermogenesis. Accordingly, it is to be expected that the BAT of fat animals exposed to

cold will be more developed (i.e. more classically recruited) because of their higher heat loss rates.

Whether or not the fat animals feel less cold is difficult to know. Perhaps the readily available deposit of energetic substrates cause the body to struggle less when performing thermogenesis, even at slightly higher heat generation levels than those necessary for leaner peers.

An alternative explanation for the fat weight association with heat conductance invokes the concept of diet-induced thermogenesis. This hypothesis states that animals receiving high-fat diets (or other hypercaloric foods) will demonstrate increased thermogenic potential (Rothwell and Stock, 1979). In the case of our mice, the only thermogenic effect of high-fat diet was observed below thermoneutrality. This detail is in disagreement with previous data acquired from measurements of UCP1 KO mice, where it has been suggested that diet-induced thermogenesis might only be visible within the thermoneutral zone (Feldmann et al., 2009). Additionally, there is some level of evidence suggesting that DIT would in fact substitute CIT (Dauncey, 1981). Therefore, a new postulation of the concept is required to explain these data.

If it happens to be the case that the higher energy expenditure of fatter animals is only seen at some level of cold exposure (i.e. at subthermoneutral temperatures), then diet-induced thermogenesis has to be a function of 1) BAT recruitment caused by the diet, and 2) the sympathetic drive received by BAT during cold exposure. In support of this theory is the fact that animals fed high-fat diet have higher O_2 consumption levels when injected with similar doses of norepinephrine (Feldmann et al., 2009). In this case, the sympathetic drive (simulated by NE injection) is the same for both groups, but intrinsic characteristics of the tissue elicit different responses.

If the last postulate is correct, then drugs that mays be able to recruit BAT could be used in association to mild cold exposure with the intent to raise energy expenditure. In fact, were this principle to be valid for humans, would obese individuals benefit from cold exposure more than leaner ones? Of course, this line of thought is meant to extrapolate the concepts to ponder upon its practical implications. The truth is that only future research will be able to provide solid answers to this question.

7.1.2 Basal metabolic rate and lean mass

While the connection between fat mass and energy expenditure can be controversial, other associations are relatively simple. This is the case for the intraspecies scaling of basal metabolic rate and lean mass (Figure 11B).

Earlier studies have characterized the proportionality between the lowest necessary costs to maintain life (BMR) and the portion of body composition that represents the metabolically-active tissues (Miller and Blyth, 1952, Cunningham, 1982). Our data come as confirmation of this principle, and even though the analyzed range of samples (20 - 27g lean mass) is relatively small, there is no doubt about the positive correlation (Paper III). The practical implication for metabolic experimentation is that basal and resting levels of energy expenditure at thermoneutrality can be rather safely normalized to the lean body mass. This would not be the case with a direct normalization to body weight, as fat mass has a low influence on BMR (Figure 11B) (Bernstein et al., 1983, Cannon and Nedergaard, 2011).

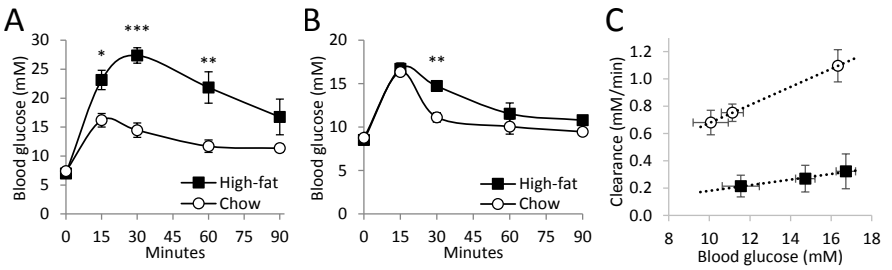
7.2 Measurement of glucose tolerance

Having defined how body composition affects calorimetry measurements, it is possible to further explore the ways in which scaling modifies the interpretation of metabolic health. Glucose tolerance test is widely used to define the capacity of an organism to clear glucose from the blood. The setup consists of measuring the blood glycaemia after a period of fasting (i.e. 6-7 h in our experiments), providing the animal with a glucose load (orally or injected), and measuring the excursion curve. When different treatment groups are compared, measured points are compared to define the speed at which values return to close-to-baseline levels. A decrease in this lowering capacity is associated with diminished tissue uptake of glucose, which in turn tends to represent an impairment in insulin signaling and diabetes.

In experimental models, it is usual to inject mice with 1 or 2 g glucose per kg body weight at the start of the experiment. It is unclear for me how this was first defined, but it is a fact that animal experimentation follows a general mentality of adjusting doses per body weight. While logical at first, this may give rise to scaling issues that affect data interpretation.

Take the example of a comparison between two groups of mice: one having received a standard chow diet, while the other a high-fat diet (Figure 12A). For the glucose tolerance test, mice were injected with 2 g glucose per kg body weight. There is a striking difference in the curves. Based on the data collected at 15, 30 and 60 minutes, it can be stated that the high-fat-fed group has a remarkably impaired glucose tolerance. Normally, the next procedure would be to calculate the area under the curve to provide numerical evidence of the difference between treatments. However, because the two groups had different initial body weights, the high-fat group received a larger dose of glucose. In this setting, the higher body weight was caused by the additional fat accumulated in the adipose tissue of mice (Figure 13). This fat is metabolically inert, but its presence causes the mice to receive a disproportionally larger dose of glucose. This can be seen by very different peak concentrations in the blood, as well as a delayed distribution of the glucose in the high-fat group (i.e. the peak is shifted towards 30 min, instead of 15). Further pharmacokinetic analysis would demonstrate a series of technical issues in this comparison. Additionally, if a group of thin mice were to be injected with higher doses, a "diabetic" phenotype would be observed (Paper IV).

In order to perform a fair comparison, a more sophisticated approach would be to give mice a dose that is scaled to their metabolically-active weight, i.e. lean mass. When the same group of mice received a dose of 1 g per kg lean mass, the resulting graphic presents an honest picture of the metabolic profile: the changed order in the glucose decay kinetics (Figure 12B). It then becomes clear that the first comparison (Figure 12A) was an exaggeration of the actual phenotype. While potentially helpful if experimenters wish to make a statement about the health of a genetically modified animal, or propose a new therapy, the setup as such is technically flawed and thus incorrect. However, when dose is normalized per lean mass, the only striking feature is one point in the curves (30 min). This is in fact extremely meaningful, and indicates that in the chow-fed group, glucose disappears from the blood with a first order kinetics: the given dose did not saturate the clearance system, and that the glucose uptake was gradient-dependent. For example, when data from points 15, 30 and 60 minutes are plotted in relation to the estimated glucose clearance at the same points, linearity is found in the chow group (Figure 12C).



D

	Dose (mg)	$T_{1/2}$	K_e (min^{-1})	C_0 (mM)	V_d (ml)	C_{max}	Clearance (mM/min)		
							15 min	30 min	60 min
Chow	25.0 ±0.8	10.6 ±1.1	0.068 ±0.007	23.2 ±1.1	6.1 ±0.4	16.0 ±0.3	1.10 ±0.12	0.75 ±0.06	0.68 ±0.09
High-fat	25.4 ±1.3	60.8 ±21.7	0.019 ±0.007	19.1 ±1.3	7.5 ±0.9	16.7 ±0.5	0.32 ±0.13	0.27 ±0.10	0.22 ±0.08
t-test	*n.s.*	**0.034**	**0.002**	**0.046**	*n.s.*	*n.s.*	**< 0.001**	**< 0.001**	**0.007**

Figure 12. Glucose tolerance tests. Mice fed chow or high-fat diet were challenged with a glucose injection of **(A)** 2 g/kg body weight or **(B)** 1 g/kg lean mass. **(C)** Correlation between glucose clearance and blood glycaemia in data points 15 and 30 min from **(B)**. **(D)** Kinetics of glucose tolerance tests performed in **(B)**. Initial glucose concentration, C_o; apparent volume of distribution, V_d; peak concentration, C_{max}; Half-life, $T_{1/2}$; elimination constant, K_e. Data points represent mean ± SEM of 4-5 mice.

On the other hand, blood glucose clearance in mice fed a high-fat diet was close to saturation. In this case, the near-linear decay (low exponential value) indicates that the uptake system is close to its maximum capacity, as estimated by the constant rate of glucose disposal (Figure 12C). The implications are then very simple: in this experiment there was a difference in the glucose clearance from the blood, and the kinetics could be properly estimated. The kinetic description of Figure 12B is presented in the table (incorporated as the Figure 12D).

The injected dose, peak glucose concentration in blood (C_{max}) and apparent volume of distribution (V_d) were similar for chow and high-fat groups. C_o represents the theoretical blood glucose concentration at minute zero, and is calculated as the y intercept of the points sampled at 15and 30 min[7]. This value was not higher in high-fat-fed mice, demonstrating that the normalized injected dose did not overestimate the capacity of mice to assimilate and diffuse glucose in the bloodstream.

[7] A natural logarithmic scale was used to linearize the exponential decay.

V_d is calculated[8] by dividing the injected dose by C_o. Chow-fed mice returned to their baseline glucose levels at a rate of circa 6.8% per min, while the high-fat group at 1.9% (presented in the Table as the elimination constant (K_e), calculated as $\ln(2)/T_{1/2}$ for each mouse). Most importantly, the increased glucose half-life ($T_{1/2}$) and saturated clearance demonstrate that in a fair comparison (i.e. glucose normalized per lean weight), mice fed a high-fat diet do not have higher glucose levels at the peak, but instead, a prolonged period of time where blood glycaemia will remain high. For the sake of exemplification, our data estimated that at the time of peak glycaemia (data point at 15 min), mice in the chow group were able to uptake 3.4 fold more glucose than their high-fat peers (Figure 12C).

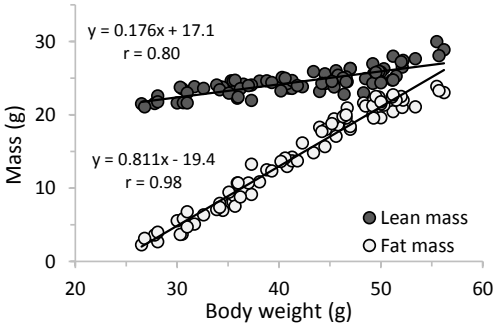


Figure 13. Correlation between components of body composition and body weight. Adult C57Bl/6 mice (over 10 weeks old), fed chow or high-fat diet were measured by magnetic resonance imaging over 6 months. While body composition constantly changed, lean and fat masses were associated to the body weight, and equations[9] were described in the graphic. While fat mass explains 81% of the body weight variation over time, lean mass accounts only for 18% of weight gain in adult mice. Data points collected from n = 10, yielding a df = 80.

In summary, the scaling of glucose dose per lean mass does not only have a sound biological rationale, but provides a fairer setup to characterize mice of different weights and allows for more sophisticated interpretation of the glucose tolerance test.

[8] The bioavailability (F) of injected glucose was estimated to be 100%, although it is arguable whether intraperitoneal injection provides the same systemic values as intravenous delivery. Because the glucose is believed to be absorbed by the mesenteric circulation, it is possible that first-pass metabolism in the liver could affect the results. For more accurate comparisons, bioavailability could be incorporated according to the following: $V_d = (F*\text{injected dose})/C_o$.

[9] The formula demonstrating the relation between lean mass and body weight can be used for the estimation of dose/lean when magnetic resonance imaging is unavailable.

8 Metabolic health

8.1 Thermogenesis in obesity

Halting the development of obesity, a condition defined by the World Health Organization as "abnormal or excessive fat accumulation that presents a risk to health", is among the biggest challenges to modern society. The development of obesity lies in an imbalance between energy intake and expenditure. An involvement of brown adipose tissue in the development of obesity has been discussed for over 35 years (Himms-Hagen, 1979, Jung et al., 1979) and recent studies have followed on the same direction (Saito, 2013) . It is important to note that the concept of defective nonshivering thermogenesis contributing to the onset of obesity was heavily influenced by the description of ob/ob mutant mice (later understood to be leptin-deficient (Zhang et al., 1994)), which were found to be hypothermic, develop obesity, and have decreased energy expenditure (Trayhurn and James, 1978). In this classic study, genetically obese mice were shown to have a lower body temperature below thermoneutrality, accompanied by decreased thermogenic potential. It had been previously shown that the same mouse model had increased sensitivity to cold exposure prior to the onset of obesity (Trayhurn et al., 1977). Therefore, a dysfunctional BAT was indicated as the causative factor of obesity. The drawback of this concept, however, is the fact that the lack of BAT thermogenesis does not necessarily cause a decreased body temperature (Golozoubova et al., 2001). Instead, the seemingly well-regulated hypothermia of ob/ob mice suggests that the hypothermic phenotype may in fact represent anapyrexia: a decreased temperature setpoint which is usually associated with energy saving. Supporting this theory is the fact that ob/ob mice do select a lower environmental temperature when given the choice (Carlisle and Dubuc, 1984). Additionally, at thermoneutrality, when the hypothermic or anapyrexic phenotype is not present (Trayhurn and James, 1978), the development of obesity in ob/ob mice is attenuated (Thurlby et al., 1978). In this case, the extra weight gained can be attributed to the hyperphagia caused by the lack of leptin. Even though it is clear that a decreased temperature setpoint will eventually lead to energy saving and obesity (if food intake in not proportionally modulated), heat generation is simply a secondary characteristic of a problem that could be characterized as neurologic. Therefore, while a genetic

modification that causes anapyrexia leads to body weight gain, there is no necessity to assume that obesity is generally caused by the lack of heat generation. The "defective thermogenesis" hypothesis, however, instigated further investigations regarding the body temperature of obese patients (conceptually summarized in (Landsberg, 2012)).

The tendency of classic studies to normalize energy expenditure data to factors derived from body weight contributed to solidify the image of obesity being caused by lowered thermogenesis (this issue is discussed in detail in (Cannon and Nedergaard, 2011)). Reality may present itself differently, though. When the levels of heat generation are directly investigated, wild type mice fed high-fat diets have generally increased thermogenic capacity, as well as slightly higher energy expenditure levels (Kuroshima et al., 1977, Feldmann et al., 2009, Cannon and Nedergaard, 2011) (Paper III).

In summary, it is still possible that confirmation bias may mislead additional studies attempting to demonstrate that BAT is less active in obese humans. This phenomenon is possible in case it becomes proven that human obesity leads to additional insulation capacity, thus decreasing the necessity for nonshivering thermogenesis, and therefore, impairing BAT recruitment. Future studies on the basic physiology of thermogenesis in obesity are necessary so that concepts can be more sophisticatedly drawn. It is probable, though, that controversy over the effects of BAT on body weight regulation will still likely remain (For further reading, I suggest (Dulloo et al., 2012)).

8.2 The paradox of healthy obesity

Perhaps one of the most astonishing aspects of metabolic research is the fact that obesity, now officially classified as a disease[10], may not be always detrimental for health. In general terms, obesity increases the risk for chronic pathological alterations, what has sparked criticism against the concept of healthy obesity (Hill and Wyatt, 2013, Kramer et al., 2013). It has been shown, however, that in certain cases obesity can be considered a protective factor against illness-related mortality (McAuley and Blair, 2011, Ling, 2013, Marques and Langouche, 2013, Lavie, 2014). The fact that

[10] Recognized by a resolution published by the American Medical Association, after lengthy discussions and voting during its 2013 Annual Meeting.

obesity could be considered "good" in the short term, but chronically detrimental, may be explained by evolutionary history. Since the capacity to accumulate energy for future use is advantageous against the eminent dangers of starvation and cold exposure, this pressure could overcome the fact that excessive energy accumulation may affect health during the second half of life (i.e. when an individual has already produced and raised offspring).

Healthy obesity may be considered a paradox partially because of scientists' failure to recognize the beneficial effects of fitness[11], which could offset the harmful effects of overweightness and complicate the prognostic evaluation of obesity (Lavie et al., 2015). Some mouse models seem to have successfully mimicked the pattern of healthy obesity, be it by the induction of genetic mutations that cause obesity without the commonly associated morbidies (Paper V and (Kusminski et al., 2012)), or by the use of drugs that cause white adipose tissue expansion in association with improved metabolic health (Paper VI and (Festuccia et al., 2008, Laplante et al., 2009)). In fact, adipose tissue has been shown to play a fundamental role in the anti-diabetic effects of rosiglitazone (Kim et al., 2003).

Additionally, genetically-modified mice rendered unable to accumulate fat demonstrate metabolic complications that could be associated with obesity (Reitman and Gavrilova, 2000). These comorbidies, in turn, can be reverted by adipose tissue transplantation (Gavrilova et al., 2000).

In summary, although the excessive expansion of adipose tissue is currently seen as a disease, the moderate accumulation of fat seems to play an important role in the conservation of metabolic health.

8.3 Energy storages and circadian cycles

Metabolic flexibility, meaning the capacity of an organism to shift metabolic pathways when necessary, has been proposed to play an important role in tissue remodeling and in counteracting the onset of cardiac and metabolic diseases

[11] Classified as the capacity of performing properly all daily activities. Therefore, an obese individual who is able to easily walk upstairs, work efficiently, and perform all household-related activities could be considered "fit".

(Taegtmeyer et al., 2004, Neglia et al., 2007, Galgani et al., 2008). Flexibility is associated with energy expenditure through different sources, such as BMR[12] (Ksiazek et al., 2009) and physical activity (Miles et al., 2009). In fact, the food composition of diets (Papers III and V), as well as circadian phase (Paper III) are fundamental to define metabolic shifts (i.e. flexibility).

In paper III, lipodystrophic ("fatless") mice have a partially disrupted circadian cycle, associated to alterations in body temperature regulation and energy expenditure. The circadian disruption could be directly linked to the low amount of accumulated fat, causing the mice to eat shorter meals over the full circadian period. It seems logical that energy stores (i.e. fat mass) could play a role in defining food-seeking-related activity in mice. This hypothesis finds support in human[13] data, where obese subjects crave less food after high levels of energy expenditure caused by intense physical activity (Thivel et al., 2012, Thivel et al., 2014).

[12] Ksiazek's study defines BMR more closely to what would be understood as RMR in this thesis. This discrepancy, however, does not preclude the use of this citation within the general concept being applied.

[13] Of note, this is an unusual situation where data gathered from human experimentation is extrapolated "backwards" to explain a phenomenon observed in mice.

9 Technical considerations on calorimetry

9.1 De novo lipogenesis

Respiratory quotient (RQ) is the dimensionless value that represents the ratio between expired CO_2 and consumed O_2. Its quantification is usually associated with the interpretation of the quality of substrates being catabolized in an animal (Ferrannini, 1988). While it does not indicate quantities, this value can be incorporated to the measurement of energy expenditure, providing an estimation of absolute rates of substrate oxidation.

Higher-than-expected RQ levels have commonly been reported as a problem for the interpretation of data and are mostly caused by unexpected endogenous conversion of glucose to fatty acids.

The calculation that follows is a theoretical experiment based on several assumptions: that the RQ value for lipogenesis is 5.55, based on basic stoichiometry (Ferrannini, 1988); that all RQ excursions above the food quotient (FQ) represent the conversion of glucose to fatty acids; and that the organism is in a state of positive energy balance. Additionally, food being provided is expected to consist of high levels of carbohydrates and relatively few lipids. This is, of course, not a situation understood as extremely common in physiology. It is applicable, though, for e.g. mice fed ad libitum with a low-fat chow diet while housed under thermoneutral conditions. Of note, RQ values may also increase when an unexpected shift towards glycolysis takes place. In the case of anaerobic glycolysis being the predominant conversion in an organism (as it is in case of high-intensity exercise), the quotient values will be higher than 1, generating errors when the interpretation of data is incautious.

All being said, the following equations constitute simply a proof-of-principle for the detection of de novo lipogenesis in indirect calorimetry systems. Therefore, they should not be referred to as an accurate estimation of glucose-to-fat conversion prior to additional validation of the mathematical principles, nor without the proper environmental conditions being met.

Assuming that the RQ values for the pure oxidation of carbohydrates (CHO), proteins and lipids are respectively 1, 0.85, and 0.7, then the food quotient (FQ) can be estimated as:

$$FQ = [CHO\% + (protein\% \cdot 0.85) + (lipid\% \cdot 0.7)]/100$$

In case the experiment would demand higher accuracy, FQ values could also be obtained by dividing CO_2 by O_2 after balancing the following equation. The values for x, y and z are based on the macromolecular substrates present in the food:

$$C_xH_yO_z + (x + y/4 - z/2)\ O_2 \rightarrow x\ CO2 + (y/2)\ H_2O$$

In order to convert the qualitative RQ value to absolute de novo lipogenesis, it is necessary to define the percentage of the whole-body metabolism (and subsequently O_2 consumption) being used for this process. The percent value was termed z. A line equation can be written as follows:

$$y = \frac{100}{5.555 - FQ} \cdot RQ - \frac{100}{5.555 - FQ} \cdot FQ$$

Followed by the normalization to 100% metabolism:

$$z = 100y / (100 + y)$$

Our unpublished results suggest that in mice housed at thermoneutrality and receiving low-fat diet, de novo lipogenesis represents on average 1.7% of the daily O_2 consumption. The next step in the calculations converts the measured O_2 consumption levels and defines the absolute amount (represented by the letter s) being used for lipid synthesis:

$$s = \frac{O2\ (L)}{100} \cdot z$$

The previously cited stoichiometry data suggest that for every 1L of O_2 used in de novo lipogenesis, 7.777 g of lipids are synthesized. Therefore:

$$x = 7.777\ s$$

When all steps are incorporated, a single equation can be written[14]. The result, represented by x, indicates the amount of triglycerides produced (g) per liter of O_2 consumed.

$$x = 7.777 \cdot \left\{ \frac{O_2\,(L)}{100} \cdot \left[\frac{100 \cdot \left[\frac{100}{5.555 - FQ} \cdot RQ - \frac{100}{5.555 - FQ} \cdot FQ \right]}{100 + \left[\frac{100}{5.555 - FQ} \cdot RQ - \frac{100}{5.555 - FQ} \cdot FQ \right]} \right] \right\}$$

Our experience has shown that this formula can be used to estimate trends of fat weight gain over long periods of time. It is, however, based on the assumption that weight gain is a linear function, while reality demonstrates that weight increases following a polynomial function that plateaus. Based on the principle that every term of this mathematical prediction has to be based on biological rationales, the lack of a clear quantifiable factor determining the natural body weight "curve" precludes the application of this equation with higher levels of accuracy.

9.2 Measurement artifacts caused by air humidity

Most calorimetry systems possess some kind of humidity removal system. In some cases, the air passes through a filter consisting of salt powders. In others, the humidity is removed by filtering through molecular sieves. This last example consists of small porous spheres, each pore being usually of the size of 3Å (necessary to trap water, but not larger molecules). Whatever system is to be used, it has to be maintained regularly. However, during long running experiments, it is not uncommon that the humidity removal system becomes saturated, altering the experimental results and data interpretation. To demonstrate how humidity affects the measurement of O_2 levels, and as a consequence, the calculation of RQ, a small theoretical experiment can be made, as following: If a mouse theoretically releases CO_2 = 1ml/min and consumes

[14] The large amount of "hundreds" makes this formulation aesthetically unpleasant. It is explainable, though, by the fact that several normalization steps have been combined. While this formulation would surely not enter the hall of "beautiful equations", its lack of elegance is compensated by brute-force functionality.

O_2 1 ml/min, the RQ value will be equal to 1. Supposing that O_2 levels in the atmosphere are 21%, CO_2 levels are 0.03%, if air flow in the calorimeter is 1 L / min, and the mouse consumes 1 ml O_2 and 1 ml CO_2 per min, then:

210 ml O_2 (basal) - 1 ml O_2 (consumed) = 209 ml O_2 (final value)
0.3 ml CO_2 (basal) + 1 ml CO_2 (released) = 1.3 ml CO_2 (final value)

This way, final O_2 will become: 20.9 % O_2 and final CO_2 will be: 0.13 % CO_2

The calorimeter will then measure consumption per minute as 1 ml O_2 and release as 1 ml CO_2. But supposing that O_2 levels in the atmosphere are 21%, and CO_2 levels are 0.03%, and humidity present in the air (H20) replaces 1% of the gases, the shifted basal levels will be:

$O_2 = 20.79$ $CO_2 = 0.0297$

If flow is 1 L / min, and the mouse consumes 1 ml O_2 and releases 1 ml CO_2 /min:
207.9 ml O_2 (basal) - 1 ml O_2 (consumed) = 206.9 ml O_2 (final value)
0.297 ml CO_2 (basal) + 1 ml CO_2 (released) = 1.297 ml CO_2 (final value)

In summary, when the system runs with incoming dry air:
210 ml O_2 basal - 209 ml O_2 measured = 1 ml /min O_2
1.3 ml CO_2 measured - 0.3 ml CO_2 basal = 1 ml /min CO_2
Respiratory quotient = 1

While results affected by humidity will be:
210 ml O_2 basal - 206.9 ml O_2 measured = 3.1 ml /min O_2
1.297 ml CO_2 measured - 0.3 ml CO_2 basal = 0.997 ml /min CO_2
Respiratory quotient = 0.32

This is, of course, a theoretical experiment where humidity yields highly discrepant measurement values. Perhaps the most important message is that sometimes, slightly decreased RQ values may be interpreted as an increase in net lipolysis, while

in reality it can be caused by a rather simple physical artifact. The same artifact would cause O_2 levels to be much higher than normal. CO_2 levels will also be affected, but in proportion to its basal presence in the atmosphere. Because the release of CO_2 is generally much higher than its normal presence in the air, the effects of humidity on CO_2 do not affect metabolic measurements (Figure 14B).

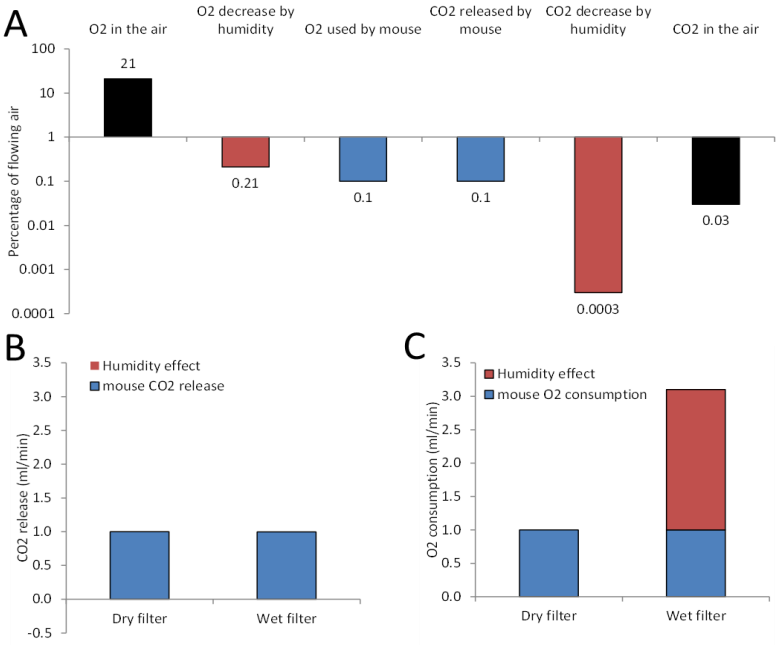


Figure 14. Effect of air humidity on indirect calorimetry measurements. **(A)** Values of respiratory gases are presented for the sake of comparison of their origins. Because of the large variation in magnitudes, values need to be presented in a logarithmical scale. **(B)** Lack of visible effect of humidity on measured CO_2 levels. **(C)** Theoretical measurement artifact caused in case humidity replaced 1% of O_2 present in the incoming air.

Therefore, when an experimenter observes an abrupt increase in O_2 consumption, this could be recognized as the first sign that the humidity-removal systems have been compromised (Figure 14C). Because this effect can be easily quantified, more advanced calorimetry systems will in fact measure the air humidity and eliminate the artifacts from the data set. Figure 14 provides a general representation of the gas measurements in the air, be them basal, metabolism-related, or humidity-related.

10 Future perspectives

In the last 3 years, over a thousand articles have been published on brown adipose tissue. Many of them attempted to describe the molecular characteristics of the tissue, others investigated its basic facets in humans.

The majority of funding supporting this research lies in the hope of developing useful therapies against obesity and diabetes. However, there are currently zero commercially-available drugs[15] (or other types of treatment) that are effective in harvesting the thermogenic potential of BAT in order to increase health. Therefore, when discussing future perspectives, it may be useful to do it with some level of self-criticism. It is surely very positive that research on thermogenesis has lately received so much support, mostly after decades of skepticism over the fact that BAT is present and active in adult humans. There is no doubt that a tissue capable of transforming glucose and fatty acids into heat has a huge therapeutic potential. Still, as a field, we have been unable to provide practical solutions for the health problems we propose to investigate.

One could argue that most discoveries are very recent, and longer time is necessary. That does not happen to be the case: the most significant advances in understanding thermogenesis as a function of BAT, as well as its potential applicability, date from the last 3 decades of the 20th century. It seems, though, that much of the classic knowledge is currently overlooked, as seen in the amount of recent studies where the proverbial UCP1 gene expression is the strongest argument to indicate thermogenesis in an organism (issue discussed in (Nedergaard and Cannon, 2013)).

It may be easy to understand, though, why old concepts are being ignored. At the present time, brown fat has become a popular subject of study also because of its capacity to attract financial support. When research focuses on capitalizing on fashionable topics, little time is left for the discussion and careful evaluation of classic knowledge. As a matter of extreme exemplification, a quick internet search will

[15] In fact, some glimpse of pharmacological hope has recently been provided regarding the activation of human BAT through β3-adrenoceptor activation (Cypess et al., 2015).

demonstrate that several patents related to BAT activation as a therapy against obesity are owned by persons who never contributed to the body of knowledge in the field.

Another present drawback in metabolic research is the idea of "finding the molecular mechanism" over "descriptive studies". While advocating a more sophisticated approach, this perspective has promoted intracellular signaling over functional evaluation, in some cases with absurd outcomes. A hypothetical example of a "mechanistic" study could be given as:

"*Molecule A binds to molecule B, causing the activation of molecule C. When molecule B is knocked out, molecule C loses function, causing disease X. When molecule B is once more expressed, molecule C regains function. Therefore molecule B represents a new therapeutic approached for the treatment of disease X.*"

This hypothetical study would have been as "descriptive" as any other, but rather questionable in sophistication and applicability. However, a quick literature search will present similar cases where high-impact studies fail to provide functional descriptions, but rely on extrapolation of data acquired in overly artificial systems. Therefore, there is a huge need for simple studies that focus on solid functional descriptions and honest applicability.

The specific activation of BAT is per se a technical challenge. Future studies will deal with the issue of targeting receptors present in BAT without causing the nonspecific activation of other tissues (what could cause unwanted side effects).

In order for thermogenesis to be used as a therapy, some considerations are necessary regarding the systemic effects to be expected. A hypothetical drug that activates brown fat will, of course, produce heat. If patients with activated brown fat were kept at thermoneutral temperatures, additional mechanisms to dissipate heat would be required to avoid hyperthermia. It may not be comfortable, though, to trigger sweating as a constant side-effect of the therapy. A solution would then be to transfer these patients to a colder environment. It is known, though, that the cooler environment would have triggered thermogenesis per se, without the necessity of a BAT-stimulating drug. This would surely raise questions regarding the effectivity and costs of the treatment. Regardless of the solution to be adopted, it is clear that therapies will have to, at some point, deal with paradoxical issues related to ambient temperatures.

A second issue is related to the possibility of behavioral compensations caused by therapies exploring heat generation. It has been proposed that BAT thermogenesis could induce weight loss, since food intake does not fully compensate the energy used for heat generation (as it is the case for intense exercise) (Cannon and Nedergaard, 2009). This is true for high levels of energy expenditure. On the other hand, minor changes in the caloric balance are generally compensated. A less optimistic perspective would consider that every form[16] of thermogenesis tends to be chronically compensated by food intake or other energy-saving adaptations (e.g. decreased spontaneous physical activity).

In summary, I hope that the studies enclosed in this thesis can provide useful steps in the construction of knowledge to be applied in the treatment of obesity and diabetes. The careful considerations on BAT blood flow (Paper I) may raise awareness on the trickiness of measuring thermogenesis, as well as be useful to avoid distractions. The characterization of central setpoints determining peripheral thermogenic features (Paper II) may provide insights into the real cause of metabolic phenotypes of animal models. The mathematical integration of body temperature (Paper III) into a greater metabolic scheme can be used in the future to more accurately evaluate whole-body energy expenditure, beyond simple abstract assumptions, toward focused calculations. The description of glucose (mis)scaling and its effects on the understanding of the health of animal models (Paper IV) could have an important role in guiding the correct evaluation of laboratory rodents and its use to study human metabolic alterations. The causality established between the presence of the protein Cidea and "healthy obesity" (Paper V) can represent an important step in the discussion of one of the most paradoxical topics in current obesity research, while the considerations on thiazolidinediones (Paper VI) may aid future therapeutics.

As a final consideration, the majority of the experiments enclosed in this thesis are based on classical physiology experimentation. In an age where experiments become increasingly expensive and overly sophisticated, it is my sincere hope that this work may stimulate simpler designs and a revival of the classic science style focused on description of function.

[16] Of course, thermic effect of food is an exception to this rule, once it is a form of thermogenesis that is directly associated to a positive caloric balance (i.e. food intake).

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