Published in final edited form as:
Comp Biochem Physiol A Mol Integr Physiol. 2011 March ; 158(3): 252–264. doi:10.1016/j.cbpa.2010.04.013.

Direct Animal Calorimetry, the Underused Gold Standard for Quantifying the Fire of Life*

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Abstract

Direct animal calorimetry, the gold standard method for quantifying animal heat production (HP), has been largely supplanted by respirometric indirect calorimetry owing to the relative ease and ready commercial availability of the latter technique. Direct calorimetry, however, can accurately quantify HP and thus metabolic rate (MR) in both metabolically normal and abnormal states, whereas respirometric indirect calorimetry relies on important assumptions that apparently have never been tested in animals with genetic or pharmacologically-induced alterations that dysregulate metabolic fuel partitioning and storage so as to promote obesity and/or diabetes. Contemporary obesity and diabetes research relies heavily on metabolically abnormal animals. Recent data implicating individual and group variation in the gut microbiome in obesity and diabetes raise important questions about transforming aerobic gas exchange into HP because 99% of gut bacteria are anaerobic and they outnumber eukaryotic cells in the body by ~10-fold. Recent credible work in non-standard laboratory animals documents substantial errors in respirometry-based estimates of HP. Accordingly, it seems obvious that new research employing simultaneous direct and indirect calorimetry (total calorimetry) will be essential to validate respirometric MR phenotyping in existing and future pharmacological and genetic models of obesity and diabetes. We also detail the use of total calorimetry with simultaneous core temperature assessment as a model for studying homeostatic control in a variety of experimental situations, including acute and chronic drug administration. Finally, we offer some tips on performing direct calorimetry, both singly and in combination with indirect calorimetry and core temperature assessment.

Keywords

metabolic rate; energy expenditure; animal heat production; homeostasis; obesity; diabetes; drug tolerance

*This submission is associated with a symposium held at the Society of Experimental Biology Annual Main Meeting 2010, Prague, entitled ‘The challenge of measuring energy expenditure: Current field and laboratory methods’. Guest Editor is Dr LG Halsey.

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Introduction

Academic and commercial interest in measuring metabolic rates has accelerated markedly in recent years. This trend reflects, in large part, the exponential growth of obesity and diabetes research (Vioque et al. 2009) spurred by the sharp rise in worldwide obesity prevalence (James et al. 2001) along with heightened interest in the role that variation in energy expenditure plays in obesity pathogenesis (Levine et al. 1999; Butler et al. 2010; Kaiyala et al. In Press).

The most accurate method for quantifying metabolic rate (MR), the direct measurement of metabolic heat generation through direct animal calorimetry, currently sees limited use in contemporary physiology and medicine. This status reflects the abundance of relatively affordable and user-friendly "turnkey" commercial systems for performing indirect calorimetry via respiratory gas exchange (respirometry), a technique that additionally provides some information on the mix of metabolic fuels being combusted. Indeed, respirometry has become by far the most common method used in research involving energy balance, including research supported by millions of dollars annually spent by the U.S. Government's National Institutes of Health (NIH) to enable metabolic characterization of the genetically altered rodent models (usually mice) that dominate contemporary cutting-edge obesity and diabetes research. By contrast, the sole commercial source we know of for direct human and animal calorimeters (Thermonetics, La Jolla CA) was not taking new orders for direct calorimeters when this manuscript was in preparation [personal communication with the inimitable Dr. Heinz Poppendiek of Thermonetics]). We contend, however, that a renaissance of direct animal calorimetry is strongly warranted given the prominent role of metabolically abnormal pharmacological, mutant and transgenic animal models in obesity and diabetes research, and a chief goal of this paper is to advance the case for this proposition. We also discuss some classic and contemporary uses of combined direct and indirect calorimetry in studies designed to better understand the homeostatic control of energy balance.

Direct Calorimetry: Definition and Overview of Utility

Direct animal calorimetry encompasses a class of techniques for measuring heat loss (HL) from subjects. HL occurs via four avenues:

- Conduction (K), heat transfer from the warmer animal to a cooler surface (e.g., floor) by direct contact;
- Convection (C), heat transfer by bulk flow of a liquid or gas away from the animal;
- Radiation (R), heat transfer via long wave [3-100 μm] electromagnetic wave energy emitted by the animal;
- Evaporation (E), heat transfer required to transform body water from the liquid to the gas phases in the respiratory passages and on skin or fur. The required heat transfer (called the latent heat of vaporization) is ~580 cal/g (~2.43 kJ/g) at room temperature.

The sum of K, C and R, is termed sensible or "dry" HL (DHL). Under typical laboratory conditions, the evaporative component of HL (EHL), often termed latent HL, mainly derives from passive evaporative water loss wherein water vaporizes from respiratory surfaces and from the skin after diffusing to this surface (IUPS 1987). Heat stress evokes autonomic (e.g., sweating, panting) as well as behavioral EHL thermoeffectors (e.g., saliva spreading in rodents) (IUPS 1987). Total HL (THL) is the sum of DHL and EHL. We stress that EHL is a sizable and variable component, representing ~10 —25% of THL, and must be quantified for accurate determination of metabolic energy transfer via direct calorimetry.

Assuming that the individual is not performing work on the environment (we make this assumption below in the heat flow balance equation), MR equals the metabolic heat production rate (HP). In the general case, MR = HP ± WR where WR is the rate at which mechanical work (force times distance per unit time) occurs. Positive WR means that the force applied by the subject displaces a mass in the direction of force application (e.g., as when lifting a weight off the floor). When WR is negative, the force applied by the subject opposes the direction the mass is moving (e.g., as when lowering the weight back to the floor). Note that an experimental subject in a calorimeter lacking specialized apparatus performs no net external work due to rearing and locomotion since the positive and negative components of such activities cancel out. Thus, in the typical calorimeter setting, MR = HP.

Rates of body heat storage and heat exchange with the environment occur in accordance with the heat flow balance equation (IUPS 1987), a statement of the First Law of Thermodynamics. Under typical laboratory conditions where E, C, K and R each mediate energy transfer from subject to environment, the heat flow balance equation may be expressed as follows:

$$S_{heat}=MR-E-C-K-R=HP-EHL\ DHL=HPTHL$$

where S_{heat} equals the rate of body heat storage (positive for body heat gain, negative for body heat loss) and the other terms are defined above.

When S_{heat} = 0, MR = HP = THL. This is a marvelously straightforward and useful result. Its only underlying “assumption” is actually a Law, namely the First Law of Thermodynamics (Conservation of Energy), according to which 100% of the chemical energy transfer associated with the innumerable energy-transformative processes of life ultimately is transformed into heat energy that flows out of the animal (for example, all chemical energy transformed into mechanical work done by the heart in pumping blood is subsequently transformed by vascular friction into heat that then flows into the environment). Accordingly, direct calorimetry is the undisputed “gold standard” for quantifying MR given sufficiently rigorous measurements with appropriate instrumentation. By contrast, indirect calorimetric methods rely on a number of violable assumptions that can produce substantial errors in HP estimates ((Walsberg et al. 2005) and discussed below).

The rationale for using direct calorimetry to quantify metabolic rate is especially easy to justify for homeotherms, i.e. animals that actively defend core temperature (T_c) within fairly narrow limits (but see below) by reflexively modulating autonomic effectors of HP and HL in response to actual or anticipated perturbations of T_c. On average, and within the zone of physiological compensation in homeotherms, S_{heat} = 0, and so THL = HP = MR. However, it should be noted that direct calorimetry has also been used to measure HP in ectotherms (e.g.,(Walsberg et al. 2006)) despite their limited repertoire of reflexive autonomic mechanisms for increasing HP. Steps should be taken to assure that such creatures are in a state of body temperature stability if the most accurate determination of metabolic HP is sought.

Importantly, the utility of the heat flow balance equation extends to non steady-state conditions, and physiologists have long measured components of the heat flow balance equation to study thermoregulatory responses in a wide variety of naturalistic settings and experimental challenges. We discuss some examples later in this paper.

A second statement of energy flow balance, one of vital importance in body energy homeostasis as it relates to body weight and obesity phenotypes is:

$$S_{chemical}=EI-MR-E_{feces}\ E_{urine}=EI-HP-WR\ E_{feces}\ E_{urine}$$

where $S_{chemical}$ equals the rate of body chemical energy storage (primarily in triglycerides, protein, glucose and glycogen, with the lipid component predominating in obesity pathogenesis), EI equals the intake rate of chemical energy in foodstuffs, and E_{feces} and E_{urine} equal the energy lost in feces and urine, respectively. We stress that obesity often arises from subtle but sustained states of positive energy balance, and therefore emphasize the vital need for accurate determination of energy balance in studies focused on the role of energy expenditure in obesity pathogenesis. Potential sources of error include the fact that E_{feces} and E_{urine} are nontrivial and potentially variable energy terms between subjects and groups, yet these components are only rarely quantified in contemporary obesity research. In this paper we present a case for why the standard assumptions used in converting respirometric gas exchange to estimates of MR may lead to substantial errors in a variety of important diabetes and obesity-related animal models. Accordingly, a major application of modern direct calorimetry will be to rigorously test key assumptions underlying the common practice of estimating MR from the rates of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) (Walsberg et al. 2005) [ref for box respirometry in this special issue][ref for mask respirometry in this special issue].

A Brief History of Direct Calorimetry

Plato, Aristotle, Hippocrates and Galen are among the early philosophers who recognized that the warmth of animals indicated the existence of an “innate fire” that sustains life (Mendelshon). However, the earliest documented measurements of the innate fire did not occur until the late 1700s. Although there is some uncertainty over who actually made the first measurements of HL (Mclean et al. 1987), priority is often given to the French chemist Antoine Lavoisier (1743-1794) and his contemporaries, including Pierre Simone Laplace (1749-1827). These pioneers assessed animal HL by measuring how much ice melted in an insulated chamber that contained both ice and the test subject (a guinea pig). Lavoisier and Laplace also estimated the amount of HL per unit of carbon dioxide production and, on the basis of separate experiments involving candles and animals, recognized that animals produce energy via a form of combustion (http://scienceworld.wolfram.com/biography/Lavoisier.html). At approximately the same time as the experiments of Lavoisier and Laplace (~1770), the Scottish scientist Adair Crawford measured HL from the increase in temperature of water in a jacket surrounding the test chamber. Crawford’s findings were published in 1777 in book wonderfully titled “Experiments and observations on animal heat and the inflammation of combustible bodies being an attempt to resolve these phenomena into a general law of nature” (a free downloadable PDF copy is available through Google Books). Crawford is credited as the first scientist to state unambiguously that oxygen consumption is proportional to HP (Blaxter 1978).

Following the pioneering calorimeter experiments of Lavoisier, Laplace and Crawford in the late 1700s, some 100 years elapsed before the next work of major significance occurred. Then, in the late 1890s, W.O. Atwater and E.B. Rosa devised a human direct calorimeter and employed it in conjunction with measurements of respiratory gas exchange in studies of enduring importance (Webb; Atwater et al. 1899). The Atwater-Rosa calorimeter consisted of a large chamber lined with copper attached to heat exchangers conveying water. The increase of temperature in a known volume of water served as the basis for calculating HL. The subject’s EHL due to respiratory water loss and sweating was quantified by passing re-circulating air through a chamber containing sulphuric acid to absorb the water (the calorimeter employed a closed-circuit design). Carbon dioxide was similarly absorbed by

potassium hydroxide (soda lime). The Atwater-Rosa system admitted oxygen into the chamber to keep pace with the subject's oxygen consumption. Accordingly, their calorimetry system enabled experiments comparing HP estimates by direct and indirect calorimetry (urinary and fecal nitrogen were also collected to estimate protein catabolism and a more rigorous accounting of carbon flow). Atwater and Benedict (Atwater et al. 1903) employed the system to conduct a storied set of experiments involving dual calorimetric measurements encompassing 150 complete 24-h days on three men (Webb 1980). These experiments earned a prominent and enduring role in the empirical justification for standard tables and formulas giving calorific equivalents of oxygen and carbon dioxide based on respiratory gas exchanges of oxygen and carbon dioxide.

Another important advance involved combining direct and indirect calorimetry with body temperature assessment. An early example of this 'total calorimetry and temperature model,' described in a classic 1935 article by Murlin and Burton (Murlin et al. 1935), was devised for human research on "heat production and heat elimination in fevers produced artificially by diathermy and by the high-frequency condensor field." (p. 234). The Murlin and Burton system involved a cylindrical respiration chamber made of Pyrex glass surrounded by an insulated kapok-like gradient layer. Dry HL across the gradient layer was calculated from the temperature differential measured by spirally-wound resistance thermometers ("gradient thermometers") that measured the temperatures on the inside and outside surfaces of the insulated layer. Air circulated through the chamber via a closed circuit design that scrubbed carbon dioxide and water vapor. EHL was calculated from the mass of water collected in bottles of sulphuric acid. Carbon dioxide was chemically absorbed (soda lime and sulphuric acid) and oxygen uptake was quantified from the volume of oxygen that was admitted into the chamber to maintain a constant oxygen level in the chamber. Rectal and skin-surface thermometers measured body temperatures. Numerous checks of the system were made using ethyl alcohol (which has a known RQ [0.67] and evolves a known amount of heat per g (7.1 kcal/g). Agreement between direct and indirect HP values, based on the alcohol checks, was impressive given the era (±2.9%, coefficient of variation = 3.6%).

An important and enduring advance in direct calorimetry occurred with the advent of Benzinger and Kitzinger's thermoelectric gradient layer calorimeter (Benzinger et al. 1949). A commercial version of the thermoelectric gradient layer calorimeter became available (Thermonetics, La Jolla, CA) based on efforts by Heinz F. Poppendiek (Poppendiek et al. 1972) and has been utilized in a number of studies (e.g., (Seale et al. 1991; Meis et al. 1994; Gordon et al. 1995; Seale et al. 1997; Gordon et al. 1998; Gordon et al. 2003; Gordon 2004)) including our own (Kaiyala et al. 2005; Kaiyala et al. 2007b; Kaiyala et al. 2007a). Room-sized human thermoelectric gradient layer calorimeters exist, including a large (3.05 × 2.74 × 2.44 m) Thermonetics-built instrument comprising the direct component of a system for synchronous direct and indirect human calorimetry at the US Department of Agriculture in Beltsville, Maryland (Seale et al. 1991; Seale et al. 1997).

Types of Direct Calorimeters

Four types of direct calorimeters exist, namely isothermal, heat-sink, convection and differential.

Isothermal direct calorimeters (also known as heat-flow or heat-conduction calorimeters (Zhang 2010)) maintain a constant wall temperature by means of a constant temperature fluid (commonly water) in a jacket or bath surrounding the animal chamber, or in a network of copper tubing bonded to an exterior wall surface (as in the Thermonetics gradient layer calorimeters and the device described in (Zhang 2010)). The best of these devices employ rigorous thermostatic control of the fluid (e.g., ± 0.01 °C)(Walsberg et al. 2005; Zhang

2010). In a thermoelectric isothermal gradient layer calorimeter, (probably the most common type of contemporary direct calorimeter) sensible heat released by the subject flows through a gradient layer "envelope" lining all surfaces of the instrument to reach the constant temperature fluid-side of the layer. In such instruments, the gradient layer is a thermopile integrated into a thin (e.g., 1-2 mm) plastic layer of uniform thickness that transforms the temperature gradient across any small area of the layer (which is proportional to the heat flow rate across that area) into a proportionate voltage signal. A thermopile consists of myriad thermocouples, each being a junction of two dissimilar metals through which current flows in proportion to the temperature difference at the junction; the thermocouples are connected in series (usually) or in parallel (sometimes). The aggregate voltage generated by the total heat flow across the entire gradient layer envelope represents the integrated heat loss due to radiation, conduction and the majority of convective loss, with the small remainder of convective HL expressed as an increase in the temperature of gas leaving the chamber.

Valuable and detailed information for building and calibrating thermoelectric calorimeters is contained in references(Walsberg et al. 2005; Zhang 2010). A formula for calculating the EHL component of THL is given in the section for direct convection calorimetry.

Heat sink direct calorimetry relies on removing the sensible heat released into the calorimeter chamber via a liquid cooled heat exchanger (usually by water circulating through a jacket in the calorimeter walls surrounding the test subject). Knowing the coolant's specific heat, flow rate, and temperature increase permit calculating the dry heat transfer from the subject. When water is the coolant, DHL in cal/min

$$DHL = \Delta T \times C_{water} \times M_{H2O}$$

where ΔT is the increase in water temperature (°C), C_{water} is the heat capacity of water (1.0 cal/[gram °C]) and M_{H2O} is the mass flow rate of water in g/min. (Note: 1.0 W ~ 14.33 cal/min for those wishing to convert to SI units).

An intriguing variant of the heat sink technique, used extensively by Paul Webb and associates (reviewed in (Webb 1995; Webb 1997)) in studies on human energy balance and thermoregulation, consists of an insulated suit calorimeter incorporating a network of thin plastic tubing through which the water flows (pictured in (Webb et al. 1972) and (Mclean et al. 1987)); a version designed to accommodate heavy exercise is described in reference (Hambraeus et al. 1994). The suit calorimeter keeps the subject in thermal comfort for long periods of time by adjusting the temperature of the influent water to match the subjects HL. Sweating is minimized by keeping the skin surface at a comfortable temperature through feedback control of the temperature of the water entering the mesh of tubing against the skin. Maintenance of a comfortable skin temperature and an absorbent suit layer minimizes EHL from the skin surface. An attractive feature of the calorimeter suit is that the subject can undergo long-term measurements of HL in just about any venue. Obviously, EHL due to respiratory water loss must be estimated or measured by a separate means.

Direct convection (also called 'air') calorimetry works by determining the temperature and enthalpy differences between the air entering and exiting an insulated chamber. The original Snellen human calorimeter (Snellen et al. 1983) and an upgraded version of this room-sized instrument (Reardon et al. 2006) are examples of convection calorimeters. In this technique, the DHL rate in cal/min can be quantified as:

$$DHL = \Delta T \times C_{air} \times V$$

where ΔT is the increase in air temperature (°C), C_{air} is the heat capacity of dry air at standard temperature and pressure (STPD; 0 °C, 1 atmosphere and chemically or mathematically (Lighton 2008) scrubbed of water vapor) (0.31 cal/[liter °C]) and V is the air flow rate through the calorimeter in l/min corrected to STPD. This equation ignores both the higher heat capacity of the water vapor in effluent air and the fact that influent and effluent flow rates are not identical, but these details may usually be ignored since water vapor constitutes < 1% of the total pressure of the effluent gas stream and the small discrepancy in flow rates is of little consequence to this calculation (Lighton 2008).

EHL in cal/min can be quantified as:

$$\mathrm{EHL}=466 \times \mathrm{V} \times (\Delta \mathrm{F}_{\mathrm{H2O}})$$

where ΔF_{H2O} is the change in the fractional concentration of water vapor and V again is the gas flow rate in l/min. Details required for more exact computations of DHL and EHL are in reference (Lighton). We note that calculations based on the formula for DHL indicate that it should be feasible to make a small animal (e.g., mouse) convection calorimeter using a 2 liter vacuum bottle as the chamber, incorporating influent and effluent ports in the lid, and employing sensitive instruments for measuring the temperature and water vapor differentials. Arranging for good distribution of air administered into the chamber (e.g., a length of aluminum tube with myriad small holes), shielding the occupant from the "wind" due to gas flow (with a baffle) and maintaining a constant temperature of the exterior walls (e.g., feedback controlled water bath) would improve accuracy and reliability. Calibration with a small electric heater would be easy. For a mouse dissipating 80% of a 0.5 W (~7 cal/min) MR via DHL and assuming a flow rate of 2 l/min, the theoretical air temperature differential at steady state is 8 °C.

Direct differential calorimetry involves two identical chambers; one housing the animal and the other an electric heater adjusted to yield identical temperature increases in both chambers. The heat supplied to the heater equates to the animal's metabolic rate.

These four types of direct calorimeters are nicely reviewed in McLean and Tobin's indispensable book (Mclean et al. 1987). We also direct attention to Paul Webb's informative reviews on calorimetry (Webb; Webb 1991) and John Lighton's treatment of the subject (Lighton).

Total Calorimetry: Old But Needed Anew

Combining direct calorimetry with simultaneous measurements of oxygen consumption and carbon dioxide production constitutes a total calorimetry model. It is worth stressing that this model is the foundation stone of our contemporary understanding of the relationship between respirometric gas exchange and metabolic rate across very broad spectrums of animal life and activity. Adding simultaneous body temperature assessment yields the total calorimetry and temperature model (e.g., (Fuller et al. 1985; Mercer et al. 1989)), so designated by Theodore Hammel and colleagues in imperishable work on thermoregulation (Hammel et al. 1963; Mercer et al. 1989; Cabanac 2006). Total calorimetry (both with and without temperature assessment) has continuing and essential roles in contemporary physiology and biomedicine. We discuss some of these below.

Role of total calorimetry in testing the assumptions of indirect calorimetry

Indirect calorimetry estimates MR based on mathematical relationships that specify the amounts of energy transfer and carbon dioxide that are liberated given the amounts and

types of metabolic fuel and oxygen that are combined, i.e., the scheme of metabolic fuel — respiratory gas stoichiometry. This scheme assigns specific but different oxygen, carbon dioxide and caloric values to each gram of a given metabolic fuel type (fat, carbohydrate or protein) that is oxidized. Accordingly, the amount of metabolic energy production per unit of oxygen uptake or carbon dioxide production depends on the proportional blend of substrates being oxidized; and estimates of substrate oxidation depend on the ratio of carbon dioxide production to oxygen consumption (i.e., the respiratory quotient (RQ)). The validity of the stoichiometry depends on meeting certain restricted conditions during testing, as discussed below. In common practice, however, the RQ is widely (often blindly) accepted as a reliable indicator of substrate oxidation and of the calorific value of oxygen. Specifically, an RQ value of 0.7 is often assumed to indicate 100% reliance on fat catabolism whereas a value of 1.0 is often assumed to indicate pure carbohydrate catabolism. The caloric equivalent of oxygen as a function of RQ is often (Swyer 1991) based on a 1928 book by Lusk (Lusk 1928) (see p.32 in (Mclean et al. 1987)), which specifies 4.69 kcal / liter O_2 when RQ equals 0.707, and 5.05 kcal / l O_2 when RQ equals 1.0. Linear interpolation is used to specify the calorific equivalents of oxygen corresponding to intermediate values of RQ. Specifically, estimated $HP = VO_2 \times (3.815 + 1.232 \times RQ)$, where HP is in units of kcal/min when VO_2 is in units of l/min. [Other formulae exist, in particular, Weir's formula is $HP = VO_2 \times (3.9 + 1.1 \times RQ)$ (Weir 1949). Note that Lusk and Weir give very similar results for a given RQ.] Many practitioners of calorimetry and probably most consumers of scientific literature featuring calorimetric data assume that RQ does not "really" deviate from the 0.7 – 1.0 range, and accept uncritically the notion that the calorific values of oxygen can be accurately specified by RQ irrespective of species, genetic status, health status and a host of other variables (Walsberg et al. 2005).

Important assumptions are made, however, when estimating substrate oxidation (and hence MR) from respiratory gas exchange (Mclean et al. 1987; Garby 1989; Simonson et al. 1990; Swyer 1991; Schutz 1997; Walsberg et al. 2005):

- Test subjects catabolize metabolic fuels with the same stoichiometry as the limited number of species represented in the original experiments (most were conducted between 1900 and 1940) that established current dogma regarding the relationships among substrate utilization, respiratory gas exchange and HP (Walsberg et al. 2005).
- Negligible net substrate interconversion occurs during the period of measurement (e.g., negligible de-novo lipogenesis from glucose; negligible ketogenesis from triglycerides; negligible gluconeogenesis from protein). This assumption is violated if, during the period of measurement, the subjects are rapidly gaining or losing body mass or in a state of poorly or uncontrolled diabetes mellitus. This assumption is violated under the metabolic conditions prevailing for some time after a meal.
- The total body carbon dioxide pool remains constant during the period of measurement. Note, however, that the carbon dioxide pool is a dynamic component of the bicarbonate buffering system that regulates body acid-base homeostasis. Accordingly this assumption is violated if the animal in a state of excess hydrogen ion production during the period of measurement, as occur during exercise and in a variety of metabolic disorders.
- The proportion of energy transfer from protein oxidation is low, essentially constant and specifiable during the period of measurement (e.g. 12.5% of the total (Weir 1949; Arch et al. 2006)). However, for those not willing to accept standard rules of thumb, protein oxidation must be quantified from excreted nitrogen. In practice, this is rarely done.

- Anaerobic metabolism plays a negligible role during the period of measurement (Lighton). Contemporary obesity and diabetes research makes extensive use of animal models that involve known or potential violations of these assumptions. Some examples are:
- A wide and growing range of animal models involving genetic alterations (spontaneous, experimentally-induced or biologically-engineered) that markedly alter metabolic processes involved in fuel handling and storage. Examples in this category include a common monogenic diabetes model (*db/db* mice) and common monogenic obesity models (*ob/ob* and A^y mice)(Barsh et al. 2002; Sindelar et al. 2002; Trevaskis et al. 2005; Tolle et al. 2008);
- Models involving pharmacological interventions that markedly alter energy balance and storage, e.g., rapid adipose tissue gain induced by pharmacological antagonism of the hypothalamic melanocortin system (Jonsson et al. 2001; Seeley et al. 2004);
- Experimental models of metabolic dysregulation. An example is streptozotocin-induced diabetes mellitus in rodents, a model characterized by marked increases of gluconeogenesis, ketogenesis, fat oxidation and energy excretion in urine (Havel et al. 2000; Sindelar et al. 2002);
- Models involving the measurement of MR during the metabolic conditions prevailing for some time after a meal wherein estimates of HP based on respirometry can deviate substantially from direct measurements of HP (Garby 1989; Garby 1991).

T our knowledge, no existing studies have examined the relationship between respiratory gas exchange and metabolic HP in any except the last of these settings. The U.S. National Institutes of Health (NIH) and a wide variety of other funding agencies annually spend many millions of dollars on research to quantify the metabolic phenotypes of genetically and metabolically altered mice and rats, and NIH funds two major mouse metabolic phenotyping core facilities (Cincinnati, Seattle; see www.mmpc.org) tasked with determining metabolic rate phenotypes in mouse and rat models used in NIH-supported research. The immense importance of models of metabolic abnormality in contemporary obesity and diabetes research would seem to represent a clarion call to test the established dogma regarding the coupling between respiratory gas exchange and metabolic HP. Other contemporary investigators have argued for re-evaluating standard beliefs regarding respirometry and metabolic HP (Webb 1980; Walsberg et al. 2005). In fact, this argument dates back to the early pioneers of combined direct and indirect calorimetry (Murlin et al. 1936). These investigators used total calorimetry to study the specific dynamic action of butter fat and sugar in humans, and reported a number of RQ values in some fat-fed subjects that fell below 0.707; in fact, they presented a formula to more accurately specify the “heat values” for oxygen based on their direct measurements of HL (the formula is 4.686 Calories per liter oxygen × [low RQ / 0.707] (p. 616). Since RQ values of 0.60 were observed, the fractional modification of the standard 4.686 kcal / liter figure can be substantial (~15%). It should be noted that the total calorimeter system was validated using 45 “heat checks” using an alcohol burner (Murlin et al. 1936). These researchers were careful.

Max Kleiber’s classic book on animal energetics, “The Fire of Life” (Kleiber 1975) includes a section entitled “Deviations from Normal RQ” (p.89). It cites studies indicating RQ values in excess of 1.0 in animals synthesizing fat from carbohydrate (“up to 1.33 in geese stuffed with grain”). John Lighton also discusses this issue in his excellent book “Measuring Metabolic Rates” (Lighton). Moreover, Kleiber actually asserted: “The RQ is inadequate as an index for the nature of the intermediary metabolism, and particularly of the precursor-product relationship.” (Kleiber 1975) (p. 93). In the next paragraph he stated: “In a study of

the relation between metabolic rate and age in [fasted] rats, we have calculated the mean results from only those trials in which the RQ (measured for a 3-hour period) was between 0.69 and 0.75." The point is that if the relationship between RQ and intermediary metabolism is uncertain, then so too is the relationship between RQ and metabolic HP. Moreover, modern scientists who ignore data from animals having RQ values at variance with the expected value may be comforted to know that they are in esteemed company.

In a historical account of energy expenditure quantification (Webb 1991) Paul Webb critiques (p. 1900) classic work of foundational importance to current respirometric indirect calorimetric dogma, noting that "A careful examination of Atwater and Benedict's classic monograph [(Atwater et al. 1903)] shows that they had recorded a discrepancy between fuel oxidized and heat loss plus work in a few experiments involving exercise and undereating, but no notice was taken because the average for all experiments showed nearly perfect agreement." Webb had previously published a paper (Webb 1980) highlighting energy balance studies that reported discrepancies between fuel oxidation and the sum of heat loss and work output.

It should be noted that spuriously low RQ values occur when water vapor in effluent gas is not adequately scrubbed or factored out mathematically (Lighton). This and other problems were avoided in carefully performed work involving combined direct and indirect calorimetry involving non-typical laboratory species (Kangaroo rat, Quail, Dove (Walsberg et al. 2005)), which nonetheless documented some RQ values well below 0.7 and some well above 1.0. Most importantly, however, was that the HP values per unit of O_2 consumption and CO_2 production in animals exhibiting normal RQ values could vary markedly from the expected HP values given by a standard formula. In Kangaroo rats, the disparity between directly measured HP and HP predicted from respiratory gas exchange was up to 38% and averaged 21%. Quoting Walsberg and Hoffman (Walsberg et al. 2005) (p. 1036): "Given the central importance of energy metabolism and the complexity of the physiological assumptions incorporated, it is striking that modern calorimetric methods have not been adequately validated. Estimates derived from indirect calorimetry have been compared to those derived by actually measuring metabolic heat production (direct calorimetry) only for conditions that were highly restricted in ways that importantly affect energy metabolism. Most of these experiments were conducted between 1900 and 1940, and primarily focused on a few species of medium-to-large mammals."

Adding to the rationale for a systematic resurgence of total calorimetry is accumulating evidence that gut bacteria constitute a metabolically significant component of the total living mass contained within human and animal bodies (Turnbaugh et al. 2006; Hildebrandt et al. 2009; Turnbaugh et al. 2009; Vijay-Kumar et al. 2010). Indeed, individual and group differences in the composition of gut microbiota are implicated in obesity pathogenesis (Turnbaugh et al. 2006; Turnbaugh et al. 2009), and experimental alterations in the composition of gut bacteria can influence both appetite and insulin sensitivity (Vijay-Kumar et al. 2010). Both obesity promoting high fat diets (Hildebrandt et al. 2009) and caloric deprivation (Crawford et al. 2009) markedly alter the microbial ecology and metabolic consequences of the gut microbiota. In particular, evidence linking variation in gut microbial ecology with differential levels of host energy harvest and storage (Turnbaugh et al. 2006; Turnbaugh et al. 2009) both suggests a potential mechanism of altered body energy homeostasis and implies that individual and group differences in microbial colonization profiles could influence measurements of whole-animal MR. Consistent with this proposition, bacteria exhibit mammal-like mass-specific metabolic rates (Gnaiger 1983; Makarieva et al. 2005) and gut bacteria are fueled almost solely by anaerobic metabolism (Guarner et al. 2003), which produces heat but is not quantified by respirometric indirect calorimetry(Gnaiger 1983). Astonishingly, the number of gut bacteria is ~10-fold larger than

the number of eukaryotic cells in the body (Bengmark 1998). The point is that individual and group differences in gut bacterial ecology could modify the whole-animal yield of heat production per unit of oxygen consumption and carbon dioxide production.

Total calorimetry and temperature as a model for studying homeostatic control during experimental challenges

Total calorimetry and temperature measurements permit systems-level studies of thermoregulatory control system behavior in a variety of experimental settings (Murlin et al. 1935; Lamprecht 1976; Roussel et al. 1979; Sugano 1983; Fuller et al. 1985; Mercer et al. 1989; Robinson et al. 1999a; Robinson et al. 1999b; Refinetti 2003; Kaiyala et al. 2005; Kaiyala et al. 2007b; Kaiyala et al. 2007a). We contend that the total calorimetry and temperature model provides unparalleled experimental access to a homeostatically-regulated variable, namely T_c and its underlying determinants, HP and HL, during acute and chronic experimental challenges (Fig. 1) (Kaiyala et al. 2005; Kaiyala et al. 2007b; Kaiyala et al. 2007a). Furthermore, diverse biological regulatory systems are proposed to depend upon similar ("conserved") architectures and protocols (Bligh 1998; Csete et al. 2002; Peper 2004). To the extent that this proposition holds true, findings based on thermoregulatory models may generalize to other regulatory domains of importance in other pathophysiological states, including obesity, which is proposed to represent a state of disordered set-point or set-point-like regulation (Corbett et al. 1986; Friedman 2009).

The total calorimetry and temperature model is perhaps most strongly associated with work by the regulatory research pioneer H.T. Hammel (Cabanac 2006) and his colleagues (Hammel et al. 1958; Caldwell et al. 1966; Schmidek et al. 1983; Mercer et al. 1989). Hammel was particularly intrigued with the nature of central homeostatic controllers. Hammel and colleagues devoted particular attention to thermoregulatory control system behavior and the nature of the thermoregulatory "set-point" using total calorimetry and temperature.

Here it should be noted that we (and many others) subscribe to the concept of "set-point" as a useful "as if" concept that need not embody a classical reference signal, but rather occurs as an emergent property of neuronal interactions involving units with different temperature sensitivities. Indeed, in 1965 Hammel proposed the original model for how neurons with different temperature sensitivities could interact so as to produce an emergent thermoregulatory set-point (perhaps better stated as the zone of minimal thermoeffector activation) without need for a reference input (Hammel 1965). John Bligh (Bligh 1979; Bligh 1998), John Boulant (Boulant 2006) and Andrej Romanovsky (Romanovsky 2007) have proposed alternative neuronal models for thermal homeostasis, with Romanovsky arguing for actuation of heat gain and heat conservation effector responses via relatively independent parallel channels, a view dating to Evelyn Satinoff's evolutionary view of thermoregulation (Satinoff 1978).

Hammel and colleagues collected data on the T_c activation thresholds and gains of thermoregulatory HP and HL effector responses using total calorimetry and temperature during experimentally controlled combinations of hypothalamic and skin temperatures. The data suggested strongly that the hypothalamic thermoregulatory set-point increased in response to lowered extrahypothalamic and skin temperatures, whereas the set point increased in response to elevated extrahypothalmic and skin temperatures (Hammel et al. 1963; Heller et al. 1978; Cabanac 2006). This pattern of system responses makes teleological sense in that if one's peripheral receptors indicate increased potential for hypothermia by sensing a cold external environment, it is homeostatically appropriate to mount heat conserving/heat producing effector responses at a higher-than-usual hypothalamic temperature and vice-versa. The resulting theoretical model was entitled

"Temperature regulation by hypothalamic proportional control with an adjustable set point" (Hammel et al. 1963), and this now stands as an accepted model for thermoregulatory control by a number of authors (e.g., see p. 390 in Refinetti's textbook, Circadian Physiology (Refinetti)). Indeed, in non-adapted dogs and monkeys (this turns out to be an important distinction, see below) the effector responses mounted by the experimental animals did not fully restore the usual (~37°C) level of T_c. Instead, during continuing periods of thermal stress, there was a persistent T_c deviation from its usual level in the direction of the thermal disturbance (e.g., T_c remained slightly lower during periods of hypothermic stress) (Hammel et al. 1963). In formal control theory, this deviation from the set-point level is termed "steady-state error," and is an intrinsic feature of control systems involving proportional control with finite gain (Nise 2004)). Hammel and colleagues (and almost everybody else in the community of Western regulatory physiologists) assumed that an ongoing deviation of the regulated variable (here T_c) from the setpoint (called a "load error") was required to drive appropriate thermal responses. However, subsequent research involving salt and water homeostasis suggested a different kind of control strategy. Specifically, Hammel and colleages found evidence that Pekin ducks regulate salt and water levels without need for an error signal (Kaul et al. 1979; Hammel et al. 1980; Hammel 1989). Indeed, in these animals, intravenous infusion of hypertonic saline evokes a robust compensatory salt secretory response that completely eliminates the steady state error that conventional thought held to be a requirement for stimulating ongoing effector responses. Hammel proposed that salt homeostasis in Pekin ducks illustrated a new phenomenon he termed "homeostasis embracing negative feedback enhanced and sustained by positive feedback" (Hammel 1989), a concept that underwent further evaluation using the total calorimetry and temperature model.

As an ardent comparative physiologist, Hammel was aware of an animal, the nine banded armadillo, which generates a *hyperthermic* T_c during periods of cold stress (Johansen 1961), suggesting an instance of "negative feedback enhanced by positive feedback" wherein the effector responses overcompensate for the homeostatic disturbance. To evaluate this possibility, it was essential to demonstrate that the armadillo generated a state of positive heat balance during cold stress, a task requiring combined direct and indirect calorimetry. Indeed, Mercer and Hammel's total calorimetry and temperature experiments (Mercer et al. 1989) established that the hyperthermic elevation of T_c was explained by positive heat storage rather than by an increase of temperature in a diminished body core volume accompanied by a net loss of body heat owing to marked body shell cooling (Mercer et al. 1989). However, perhaps the most interesting finding was that the effector responses actually drove body heat storage (and T_c) not just back to, but well beyond the usual level (by an average of 1.3°C). "Why body core temperature in these cold-exposed animals should increase at all seems puzzling … the animals maintained the increase in metabolic heat production and in body core temperature throughout the 3-h exposure period. It would seem that during cold exposure the animals were regulating their body core temperature at an elevated level." (p. 588). It appears that little work relevant to the issue of regulatory overcompensation occurred over the next decade. Then came a smattering of results bearing intriguing analogy to Hammel's armadillo. In carefully controlled experiments involving rats housed in cold (12°C) ambient temperature, Yang and Gordon (Yang et al. 1996) found that rats maintained an elevated T_c. In separate research involving cold-adapted rats (housed for > 1 month at 3-5°C)(Szekely et al. 2001), a paradoxical increase of T_c (described by the authors as an "overshoot") was documented when the rats were abruptly exposed to intense cold. Moreover, the metabolic rate of the cold-adapted rats increased immediately upon cold exposure, and did so in the absence of any initial decrease of T_c. Again, the conventionally required error signal was missing, and the temperature set point behaved as if it abruptly adjusted upwards.

Later, in 2005, our group reported analogous findings in rats given repeated exposures to the inhalant nitrous oxide (N2O).

Initial administrations of certain drugs, notably nitrous oxide (N2O) or ethanol, evoke hypothermia, but with repeated administrations, the magnitude of hypothermia wanes, consistent with the development of tolerance. It is worth noting that ethanol-induced hypothermia became a mainstay model for studies on the etiology of alcohol tolerance (Palmer et al. 2002; Lovinger et al. 2005). Initial exposure to ≥60% N2O maintained as a steady state "clamp" also evokes hypothermia in rats (Quock et al. 1987; Kaiyala et al. 2007a), and sub chronic repeated exposure to this inhalant results in "classical" tolerance to the hypothermic effect (Ramsay et al. 1999). We say "classical" because drug tolerance is conventionally thought of as a state wherein the monitored variable remains at or just slightly different from baseline during drug administration, with any difference being in the direction of the original drug effect (Ramsay et al. 1997). A nice thing about this model is that N2O is easily administered as a clamped (and almost square wave) drug input, since the amount of N2O dissolved in body fluids is determined solely by the inspired concentration and because N2O is, for all practical purposes, not metabolized in the body. Accordingly, tolerance cannot be explained by diminishing drug levels due to pharmacokinetic mechanisms. Unexpectedly, however, it turned out that administering more than the usual sub chronic number of exposures to 60% N2O resulted in a significant *hyperthermic* overshoot during administration (Ramsay et al. 2005).

We developed our contemporary extension of the total calorimetry and temperature model (with generous support by NIH/NIDA) to better understand the underlying basis for initial drug sensitivity, drug tolerance, and to clarify the overshoot phenomenon.

Initial drug sensitivity—A long-standing but controversial model of initial drug sensitivity and tolerance, one that we term the adaptive/regulatory model, predicts that a drug's primary pharmacological effects trigger homeostatic corrective regulatory responses of varying strength even during initial administrations. If so, some commonly used *in vivo* measures of initial drug sensitivity (such as T_c) are difficult to interpret in isolation because their values represent the summation of underlying inputs. To our knowledge, this adaptive/regulatory model had never been tested in a direct fashion. Our model allowed us to conduct experiments to determine if T_c stability during drug administration can mask variability in both the underlying pharmacological effects and in the physiological counter-responses during an initial administration of the hypothermia-promoting drug, N_2O.

Drug-naïve rats each received a 90-minute exposure to 0, 15, 30, 50, 60, or 75% N_2O in addition to a paired, control gas exposure (n ≥8 per group). Rats were randomized to dose group, and the paired exposures occurred in counterbalanced fashion 7 days apart.

We found that T_c was unaltered by concentrations ≤50% N_2O, but at 30 and 50% N_2O this stability masked significant increases of HL that were offset by increases of HP. The relationship between N_2O dose and HL was approximately linear while the relationship between N_2O dose and HP was an inverted U (Fig. 2A). Therefore, on average, hypothermia accompanied 60 and 75% N_2O inhalation owing to uncompensated increases of HL. However, some rats administered these higher N_2O doses also exhibited T_c stability via significant opposing changes of HL and HP (Fig. 2D). In summary, measurements of T_c in rodents can fail to disclose underlying pharmacological sensitivity owing to regulatory counter-responses. This finding is important, in part, because T_c assessment is a very common *in vivo* measure of initial drug sensitivity and sensitivity to toxicants (Gordon 2005; Gordon et al. 2008).

Drug tolerance development—Typically, 60% N_2O initially lowers T_c, but hypothermic tolerance develops with chronic administration. Therefore, one or both of T_c's controlling determinants, HP and HL, must adapt across repeated N_2O administrations.

Simultaneous measurements of HP, HL and T_c were obtained during 60% N_2O administrations in adolescent (28-45 days, n=11) and mature rats (>90 days, n=8). Each rat received five consecutive 210-min gas exposures, one per day on an every other day schedule. T_c, HP and HL were quantified throughout each exposure. Each gas exposure entailed an initial 120-minute control gas exposure (21% O_2:79% N_2) followed seamlessly by a 90-minute N_2O exposure (60% N_2O:21% O_2:19% N_2).

Compared to mature rats, adolescent rats initially exhibited greater hypothermia (Fig. 3A, C), but acquired tolerance more rapidly and actually developed an allostatic-like *hyper*thermia by the 5th administration (Fig. 3B, C). In both groups, N_2O consistently increased HL (Fig. 3E, F), but progressive increases of intrasessional HP over repeated administrations (Fig. 3I) prevented hypothermia and subsequently promoted *hyper*thermia in adolescent rats (Fig. 3B). A subset of 4 of the 11 adolescent rats received 6 additional every-other-day N_2O exposures for a total of 11 N_2O exposures. They continued to adapt to additional N2O exposures with a further development of an allostatic-like hyperthermia. The difference between the hyperthermic magnitudes during Session 11 and Session 5 was significant ($p < 0.05$) and the drug's effect to increase HL persisted. In the 11th exposure, T_c increased from baseline by an average of 0.93°C ($p<0.001$).

In summary, acquired increases of intrasessional HP across N_2O administrations explained both tolerance to N_2O hypothermia and the unexpected *hyper*thermia observed in adolescents, a finding that challenges the traditional view of tolerance. This view holds that in the fully tolerant state, the monitored dependent variable will remain close to baseline during drug administration, and will not exhibit a sign-reversal over-correction (Dworkin 1993). We have since replicated and extended the hyperthermic sign-reversal outcome in a larger cohort of rats (n = 40) subjected to twelve 70% N2O exposures. We contend that this line of research is important, in part, because sign-reversals of homeostatic variables engendered by chronic drug use are proposed to result in motivated states that promote increased drug taking behavior, a proposition known as the allostasis model of addiction (Koob et al. 2004). The allostasis model predicts that if given the behavioral means to alleviate the hyperthermic T_c during N2O administration, rats will do so. This hypothesis is currently being tested using a custom gas-tight thermally-graded alleyway in which rats that have developed a hyperthermic overshoot of T_c due to repeated drug exposures are allowed to select their preferred ambient temperature during N2O administration.

Tips on Performing Direct Calorimetry Singly and in Combination with Indirect Calorimetry

Calibrate frequently

Frequently check your direct calorimeter using an appropriate electric heater and lab power supply to generate heat inputs equivalent to the range of HL generated by your subject animals. A nice feature of direct calorimeters is that they are easy to calibrate with respect to sensible HL.

Maintain a constant wall temperature

It is essential to maintain the external walls of the gradient layer envelope of an isothermal gradient layer calorimeter at as constant a temperature as possible. Calibrate the gradient layer calorimeter at the temperature used during animal studies. Do not uncritically apply the manufacturer's calibration constant if using a commercial system. If the wall temperature drifts, so does the voltage signal output generated at a constant heat input in the

calorimeter. We utilize a plastic 100 gallon water tank (US Plastics) whose thermal mass keeps the water temperature very constant at the mean temperature of the laboratory. Immersed in this large mass of water is a heat exchanger comprised of 100 feet of coiled copper tubing. This conveys water from the source tap to, and through, the serpentine network of copper tubing affixed to the outer walls of our Thermonetics gradient layer calorimeters. Moreover, before the water passes into the big water tank/heat exchanger, it flows though an automobile radiator. This component turned out to be vitally important in our laboratory because in the winter, our tap water gets rather cold (~7°C), and unless it is pre-warmed to room temperature, its flow through the water tank's heat exchanger will lower the temperature of the water bath. The radiator does a splendid job of bringing the tap water to laboratory temperature, and the heat exchanger very effectively brings the temperature to that of the water in the tank. Our most vexing problem has been to convince the building authorities of the need to maintain our laboratory's temperature within narrow limits, 22 ± 1°C absolute. A friendly persistence is essential. We collect data on laboratory temperature and the temperature of the water supplying the calorimeters throughout all studies.

The water inflow rate into the calorimeter's wall plumbing must be regulated carefully. We utilize a custom flow regulator at the common wall tap that supplies a manifold having six simple flow regulators (Dwyer, cat. no. VFA34SSV). We set these to regulate the inflow rate at 100 ml/min. After passing through the calorimeter wall plumbing, the water passes into a common floor drain.

Measuring EHL

EHL comprises ~10-25% of total HL and thus is a quantitatively important component. For studies requiring a fine level of temporal resolution in EHL, use a high quality precision relative humidity dewpoint sensor with a fast response time (e.g., Sable Systems RH-300). Less sophisticated devices are less precise and have a slow response time, which distorts the temporal profile of EHL.

We place either a custom animal container or a custom support structure in the calorimeter to prevent urine and feces from wetting the animal during testing and to prevent access to internal antenna components. The animal resides on parallel plastic rods spaced so to allow waste to drop to a plastic tray immediately below the animal. We have utilized containers/support structures of varying design. One version has side-walls that extend almost to the top of the calorimeter chamber, but are amply perforated to provide a minimal barrier to heat transfer. Another version entails a very short side-wall designed with a lip that extends to the calorimeter walls to prevent the animal from chewing on it and to prevent urine/feces from going over the side and compromising antenna components positioned on the calorimeter floor.

Urine from the test animal yields a source of EHL that can reflect some non-animal-heat derived evaporation unless the urine output is isolated. This task can be accomplished by placing a pan containing a thin layer of mineral oil below the animal. Since water is heavier than the oil, the urine sinks below the oil, which prevents evaporation derived from energy contained by influent gas flow *per se* rather than by the animal. However, mineral oil is very messy to work with, and if spilled is detrimental to the intra-chamber components of the T_c measuring system. Mineral oil readily gets on the animal and other surfaces within the calorimeter when the support grid allows the animal's tail to dip into the oil. We are still working on better ways to isolate urine output.

Improving simultaneous core temperature assessment

The direct calorimeter's metal walls require an intra-chamber placement of the custom antenna used to acquire the T_c signal from the animal's intraperitoneal sensor (previously the MiniMitter VM-FH, Bend, OR; currently Data Sciences International, St Paul, MN). In our experience with this configuration, grounding the exterior metal shell markedly improves the quality of telemetric T_c signals.

Improving simultaneous indirect calorimetry

Since we administer custom gas blends, we use push-mode respirometry (Lighton) in conjunction with direct calorimetry. Push-mode respirometry necessitates a gas-tight calorimeter chamber, which can be surprisingly difficult to achieve. We use a generous amount RTV silicone to seal the interior walls. To provide an effective seal between the lid and body of the calorimeter, six cam-type latches and a custom gasket (currently product G-207-N from Rubatex International, Bedford, VA) are retrofitted to each calorimeter. We determine how well each calorimeter is sealed by comparing incurrent gas flow rates (based on the mass flow valves that blend influent gas for each calorimeter) to effluent gas flow rates (based on a mass flow meter).

For systems not requiring custom incurrent gas blends, we suggest considering the use of pull-mode respirometry, because this approach utilizes a non-sealed calorimeter chamber (Lighton). It is well worth knowing that HP can be estimated from VO_2 without using RQ or assuming a particular value of RQ (e.g., 0.85), a point highlighted by Arch and Speakman in their excellent review (Arch et al. 2006). The formula, based on Weir (Weir 1949), can be expressed as follows:

$$\mathrm{HP(kcal/min)} = 5.0 \times \text{fractional } O_2 \text{ delta} \times \text{effluent gas flow rate(l/min)}$$

where fractional O_2 delta is the difference in the fractional O_2 concentration between influent and effluent gas. Based on the information in (Arch et al. 2006), we derived the above formula in terms of the influent gas flow rate and found that one can substitute the influent flow rate for the excurrent rate in the formula. Assuming that the standard assumptions regarding the relationships between respirometric gas exchange and HP are valid, the above formula estimates HP with an error that is "less than one in 600 when fat is the fuel and approaches zero as the proportion of carbohydrate increases." (p. 3 in (Arch et al. 2006)). This formula provides an attractive way to estimate HP in studies wherein CO_2 is not measured.

Concluding Comments

Given the immense reliance on metabolically abnormal genetically-altered and other animal models in contemporary obesity and diabetes research, and given recent work involving non-standard laboratory animals that challenges assumptions used in respirometric indirect calorimetry, it seems obvious that new total calorimetric studies are vitally needed to validate those assumptions in select animal models. In addition, the total calorimetry and temperature model has much untapped potential for studying the homeostatic control of energy balance in a wide variety of experimental settings, including acute and chronic drug administration, and obesity pathogenesis.

Acknowledgments

This paper is dedicated to the late Shehzad Butt, Research Scientist Extraordinaire. Supported by NIH grants DA023484, U24 DK076126 and 20497-22/U24DK076169-03.

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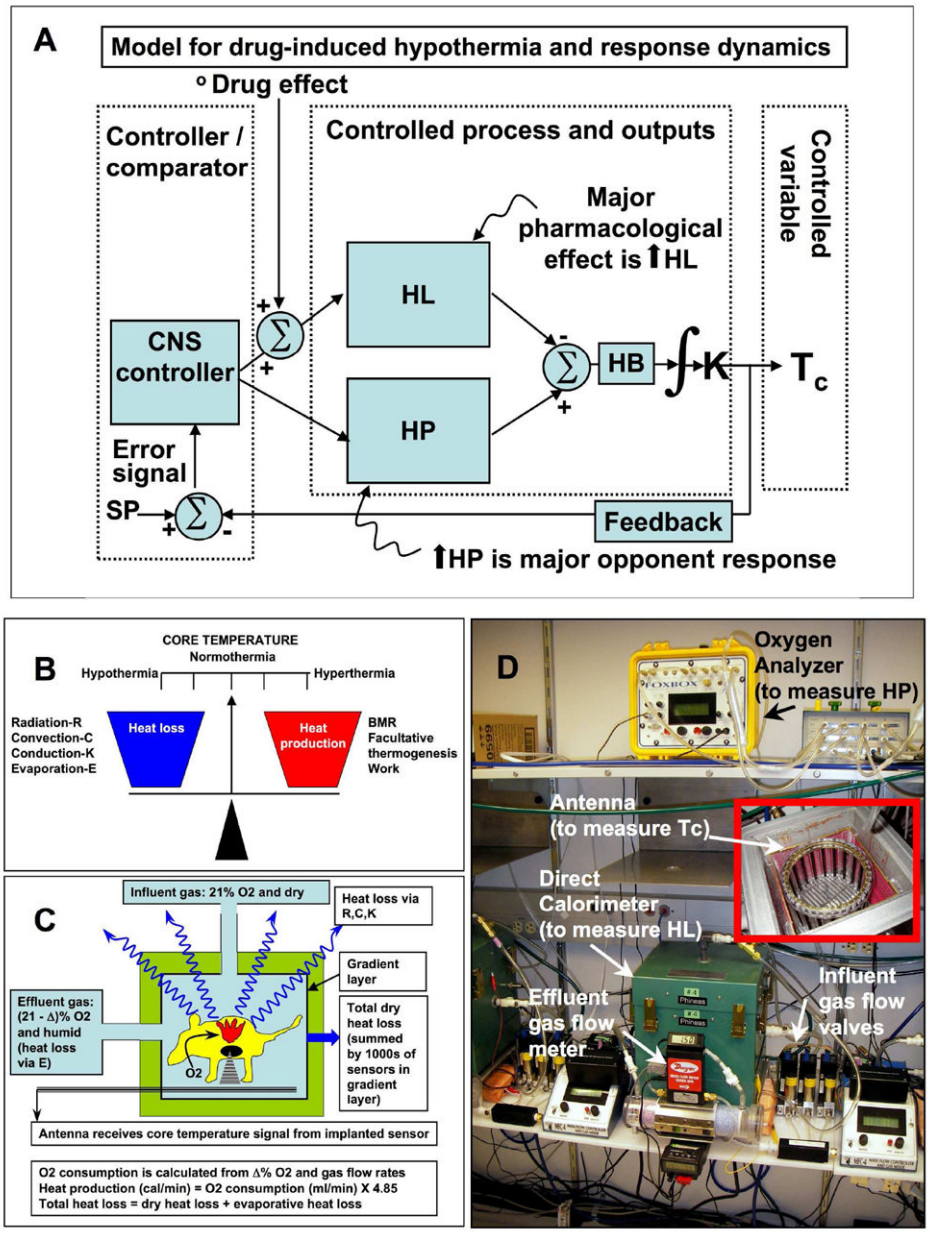

Figure 1. Total calorimetry and temperature as a model for investigating homeostatic regulatory system adaptations to drug administrations

The control system for Tc is schematized in panel **A** using control theory terminology. A CNS controller /comparator (leftmost dashed box) receives feedback from Tc, the controlled (regulated) variable (rightmost dashed box). A comparator of unknown design but shown in classical form for simplicity generates an error signal when Tc deviates from a set-point (SP, typically ~37 °C). The controller responds to the error signal by issuing corrective actuating commands to the controlled process (center dashed box). This process includes mechanisms that govern heat loss (HL), e.g., tail blood flow, and heat production (HP), e.g., brown fat. The sum of corrective changes in HL and HP constitutes a corrective change of heat balance (HB) which upon integration (integral sign) causes a change in body heat content that, when expressed in accordance with tissue specific heat and body mass (K) constitutes a corrective change in Tc. When a “hypothermic” drug is introduced into a system at normal temperature

equilibrium, the primary effect of the drug (^{o}Drug effect) is to abruptly increase HL (shown as doing so by summing with the HL actuating signal in accordance with the classical control systems representation of a disturbance acting to perturb a controlled process (see (Nise 2004), p. 388)). The ensuing decrease of Tc acts via the feedback loop to elicit corrective responses, expressed mainly as increased HP in our experiments (Kaiyala et al. 2007a; Kaiyala et al. 2007b). In reality, the control system adapts over repeated administrations to become highly effective in defending Tc homeostasis during drug administration despite continued pharmacological efficacy in promoting increased HL. However, in some animals, the adaptation in HP becomes excessive and allostasis ensues (see Fig. 4). (Note: we acknowledge that this model is overly simplified and ignores other possible sites of drug action and possible interactions with other homeostatic systems. Limited space precludes much discussion beyond this, but see (Kaiyala et al. 2007a; Kaiyala et al. 2007b)). Importantly, all controlled-process mechanisms (effectors) for Tc necessarily act by modulating rates of HL or HP, a fact emphasized by panel (**B**), which also specifies the avenues of HL and HP. Panel (**C**) illustrates the essential features of total calorimetry and temperature measurements. The heat released from the rat (squiggly lines) is measured via myriad thermoelectric sensors located in the gradient layer of the direct calorimeter (green box). All heat flow across the gradient layer, whether derived from conduction, convection or radiation, is by convention lumped into “dry heat loss” (DHL). Evaporative HL is measured based on the humidity added to the effluent gas. HP is measured from oxygen (O_2) consumption (O_2 fuels the rat’s “fire of life,” a term coined by the eminent physiologist Max Kleiber, with a specified relationship between O_2 uptake and heat production (1 ml O_2 ~ 4.85 calories). Tc is measured via a sensor implant (DSI, Inc.), which broadcasts the temperature signal to an antenna. Panel (**D**) shows one of our six systems. The inset shows the loop antenna and animal container. Note the three mass flow valves for blending influent gas mixes, including 21% O_2:79% N_2 (custom air) and 0-79% N_2O blended with 21% oxygen (balance N_2).

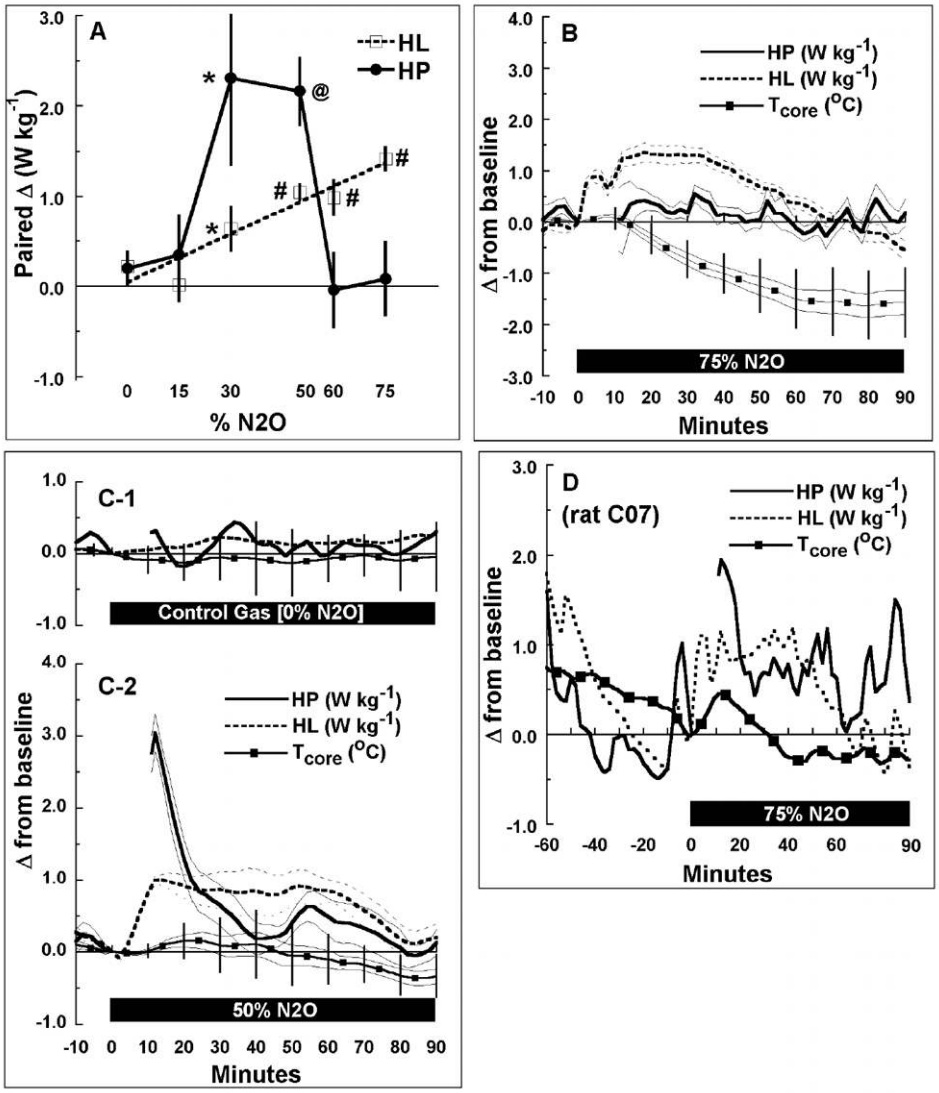

Figure 2.

A: Dose-dependent paired changes (N_2O minus control) of HP and HL during the initial period of N_2O administration (means over minutes 10 – 20 after N_2O onset). Eight rats per group except n = 11 for 60%. The important point is that the underlying determinants of T_c are significantly altered at concentrations of N_2O (30 and 50%) that exert no net effect on T_c. Bars indicate standard errors. * = $p<0.05$; @ = $p<0.001$; # = $p<0.0001$. Baseline thermal values did not differ between control and drug exposures. **B:** Thermal changes during administration of 75% N_2O. Hypothermia occurred via a significant increase of HL. This pattern also occurred with 60% N_2O (not shown). The thin continuous lines indicate standard error bands. The vertical lines bracketing T_c at 5-minute intervals indicate standard deviations to depict the individual variability in the hypothermic magnitude at this high drug dose. Control and N_2O baseline values at Minute zero did not differ significantly. **C:** Thermal changes during administration of control gas (panel C-1) or 50% N_2O (Panel C-2) in the same 8 rats. The ΔT_c profiles are nearly identical, but the maintenance of normothermia during 50% N_2O did not reflect insensitivity to this concentration, but instead occurred because a significant increase of HL was nullified by increased HP. The thin continuous lines indicate standard error bands (not depicted for C-1). The vertical lines

bracketing T_c at 5-minute intervals indicate standard deviations to depict individual variability. Control and N_2O baseline values at Minute zero did not differ significantly. **D:** Thermal changes during administration of 75% N_2O to one rat that appeared to be insensitive to this high concentration at the level of T_c. Note that the rat did appear to be sensitive to the drug's HL effect given the sustained (40-minute) increase of HL averaging 0.96 W/kg (14 cal/kg/min) starting with the onset of drug administration, but this effect was almost perfectly matched by an opposing increase of HP with similar temporal characteristics. Data adapted from (Kaiyala et al. 2007a).

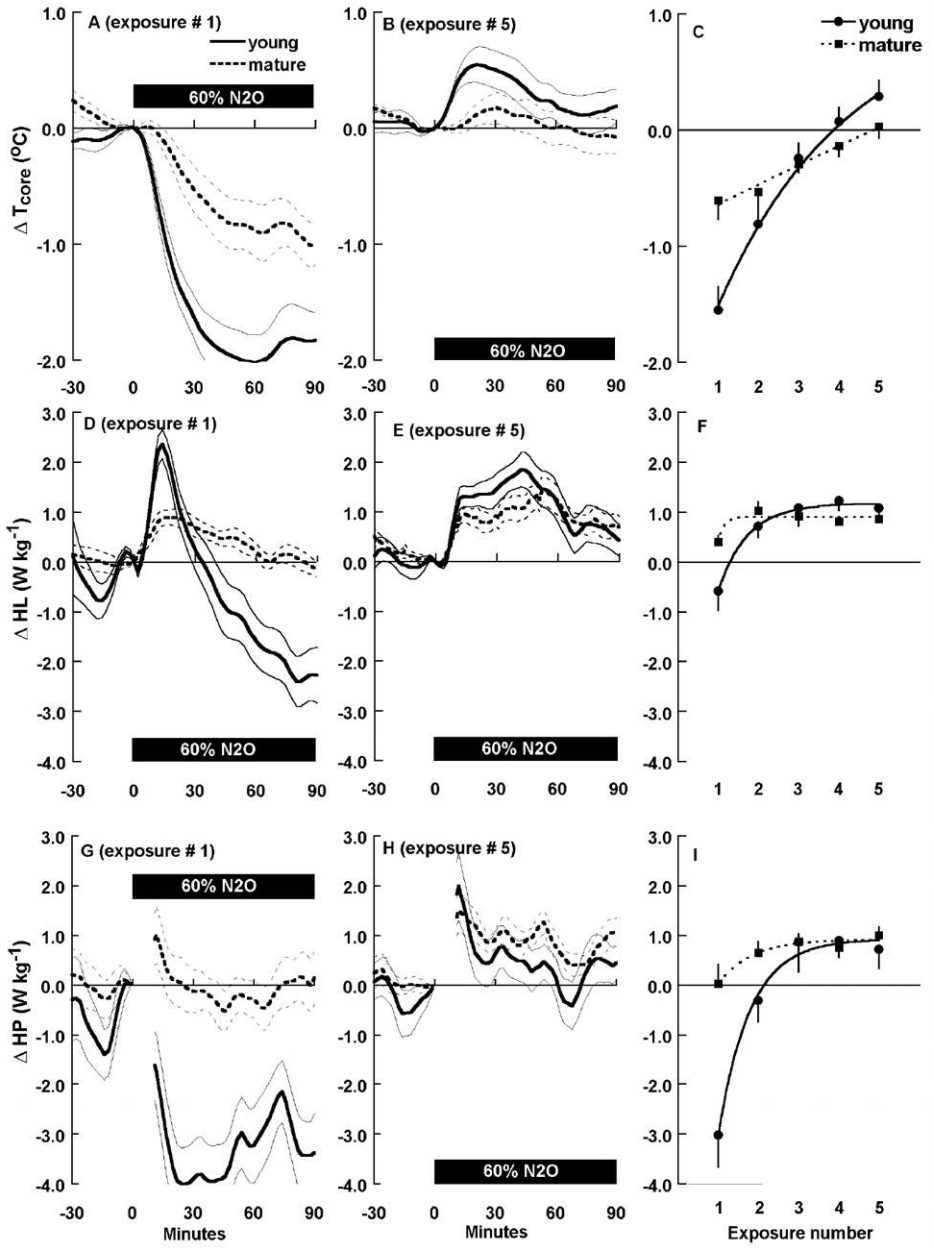

Figure 3.

Changes of T_c and its controlling determinants during repeated N_2O administrations in adolescent and mature rats. The left two columns depict group comparisons of thermal profiles with standard error bands in the first and fifth exposures, respectively. The right column shows the group by session 90-min mean changes from pre-drug baseline. Note that adolescent rats were initially very sensitive to N_2O hypothermia but rapidly developed tolerance and then a hyperthermic overshoot with repeated exposures. Bars indicate standard errors. Data adapted from (Kaiyala et al. 2007b).