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TITLE:

Determining basal energy expenditure and the capacity of thermogenic adipocytes to expend energy in obese mice

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SUMMARY:

This manuscript describes a protocol to measure the basal metabolic rate and the oxidative capacity of thermogenic adipocytes in obese mice.

ABSTRACT:

Energy expenditure measurements are necessary to understand how changes in metabolism can lead to obesity. Basal energy expenditure can be determined in mice by measuring whole-body oxygen consumption, CO_2 production, and physical activity using metabolic cages. Thermogenic brown/beige adipocytes (BA) contribute significantly to rodent energy expenditure, particularly at low ambient temperatures. Here, measurements of basal energy expenditure and total BA capacity to expend energy in obese mice are described in two detailed protocols: the first explaining how to set up the assay to measure basal energy expenditure using analysis of covariance (ANCOVA), a necessary analysis given that energy expenditure co-varies with body mass. The second protocol describes how to measure BA energy expenditure capacity *in vivo* in mice. This procedure involves anesthesia, needed to limit expenditure caused by physical activity, followed by the injection of beta3-adrenergic agonist, CL-316,243, which activates energy expenditure in BA. These two protocols and their limitations are described in sufficient detail to allow a successful first experiment.

INTRODUCTION:

Metabolism can be defined as the integration of the biochemical reactions responsible for nutrient uptake, storage, transformation, and breakdown that cells use to grow and perform their functions. Metabolic reactions transform the energy contained in nutrients into a form that can be used by cells to synthesize new molecules and execute work. These biochemical reactions are inherently inefficient in transforming this energy into a usable form to sustain life[1]. Such inefficiency results in energy dissipation in the form of heat, with this heat production being used to quantify the Standard Metabolic Rate (SMR) of an organism[1]. The Standard condition was classically defined as heat production occurring in an awake but resting adult, not ingesting or digesting food, at thermoneutrality and without any stress[1]. The Basal Metabolic Rate (BMR) or basal energy expenditure in mice is referred to as the SMR but in individuals ingesting and digesting food under mild thermal stress (ambient temperatures 21-22 °C)[1]. However, the challenges and difficulties of directly measuring heat production by oxygen consumption measurements, known as indirect calorimetry, have become the most popular approach to determine the BMR. Calculating the BMR from oxygen consumption is possible because the oxidation of nutrients by mitochondria to synthesize ATP is responsible for 72% of the total oxygen consumed in an organism, with 8% of total oxygen consumption also occurring in mitochondria but without generating ATP (uncoupled respiration)[1]. The majority of the remaining 20% of oxygen consumed can be attributed to nutrient oxidation in other subcellular locations (peroxisomal fatty acid oxidation), anabolic processes, and reactive oxygen species formation[1]. Thus, in 1907, Lusk established an equation, based on empirical measurements, widely used to transform oxygen consumption and CO_2 production into energy dissipation as heat. In humans, the brain accounts for ~25% of the BMR, the musculoskeletal system for ~18.4%, the liver for ~20 %, the heart for ~10%, and the adipose tissue for ~3-7%[2]. In mice, the tissue contribution to BMR is slightly different, with the brain representing ~6.5%, the skeletal muscle ~13%, the liver ~52%, the heart ~3.7%, and adipose tissue ~5%[3].

Remarkably, the biochemical reactions defining the BMR are not fixed and change in response to different needs, such as external work (physical activity), development (tissue growth), internal stresses (counteracting infections, injuries, tissue turnover), and changes in ambient temperature (cold defense)[1]. Some organisms actively recruit processes to generate heat in cold exposure, implying that heat produced by metabolism is not just an accidental byproduct. Instead, evolution selected regulatory approaches that could specifically upregulate heat production by changing the rate of metabolic reactions[1]. Thus, these same oxygen consumption measurements can be used to determine the capacity of an organism to generate heat in response to cold.

Two major processes contribute to heat generation upon cold exposure. The first one is shivering, which generates heat by increasing mitochondrial oxidative phosphorylation and glycolysis in muscle to cover the physical work done by involuntary muscle contraction. Therefore, cold exposure will increase oxygen consumption in muscles[1]. The second is Non-Shivering Thermogenesis, which occurs through an increase in oxygen consumption in brown and beige adipocytes (BA). Dissipation of energy into heat in BA is mediated by the mitochondrial uncoupling protein 1 (UCP1), which allows proton re-entry into the mitochondrial matrix, decreasing the mitochondrial proton gradient. The dissipation of the mitochondrial proton

gradient by UCP1 increases heat production by the elevation in electron transfer and oxygen consumption and the energy released by proton dissipation *per se* without generating ATP (uncoupled). Moreover, thermogenic BA can recruit additional mechanisms that elevate oxygen consumption without causing a significant dissipation in the proton gradient by activating futile oxidative ATP synthesis and consumption cycles. The metabolic cages described here, namely the CLAMS-Oxymax system from Columbus Instruments, offer the possibility to measure energy expenditure at different ambient temperatures. However, to determine BA thermogenic capacity using whole-body oxygen consumption measurements, one needs to: (1) eliminate the contribution of shivering, and other non-BA metabolic processes to energy expenditure, and (2) specifically activate BA thermogenic activity *in vivo*. Thus, a second protocol describes how to selectively activate BA *in vivo* using pharmacology in anesthetized mice at thermoneutrality (30 °C), with anesthesia and thermoneutrality limiting other non-BA thermogenic processes (i.e., shivering). The pharmacological strategy to activate BA is treating mice with the β3-adrenergic receptor agonist CL-316,246. The reason is that cold exposure promotes a sympathetic response releasing norepinephrine to activate β-adrenergic receptors in BA, which activates UCP1 and fat oxidation. Furthermore, β3-adrenergic receptor expression is highly enriched in adipose tissue in mice.

PROTOCOL:

All experiments were approved by the Institutional Animal Care and Use Committee at the University of California, Los Angeles (UCLA). Mice were administered their diet and water *ad libitum* in the metabolic cage, housed in a temperature-controlled environment (~21-22 or 30 °C) with a 12h light/dark cycle. 8 week-old female mice fed a high-fat diet or chow diet for 8 weeks were used for this study.

1. Measurement of the Basal Metabolic Rate (BMR)

1.1. Measure the total bodyweight of the mouse using a weight scale with accuracy in the range of 0.1 g.

NOTE: This must be done before housing the mice in metabolic cages and after the 2-3 days of acclimation period to the metabolic cages.

1.2. Measure the body composition, including fat and lean mass in non-anesthetized mice, using an appropriate body composition analysis system (see **Table of Materials**).

NOTE: These measurements are needed to determine energy expenditure and are executed parallel to total body weight measurements (step 1.1).

1.3. Set up the metabolic cages and start the acclimation period.

NOTE: The metabolic cages system includes an enclosure that allows the user to control the housing temperature and light of 12 cages (**Figure 1A, B**). Each cage has a water bottle, a feeder, and a grid (**Figure 1C**). The grid separates the mouse from the bottom of the cage, allowing feces

collection. Once the cage is installed on each pre-determined space, a lid that seals the cage includes the water bottle slot, the tubing sampling air, the airflow system, and the physical activity sensor (**Figure 1D**).

1.3.1. Turn on the temperature enclosure, the airflow system, and computer 2 h before starting the assay.

1.3.2. After 2 h, open the software controlling the enclosure (see **Table of Materials**) and the airflow and let the software test the computer's communication with the equipment.

NOTE: Oxymax software was used for the present work.

1.3.3. Once communication is established, click **File**, then **Open Experiment Configuration** (**Figure 2A**) and select the experiment configuration predesigned by the vendor (or set up from a previous assay).

1.3.4. Click on **Experiment**, then click on **Properties**, which will open the Experiment Properties window (**Figure 2B**).

1.3.5. In the Properties window, set up the parameters of the environmental enclosure, including the Ambient Temperature (21 °C, with contribution from thermogenic BA) and the 12 h Light cycles.

NOTE: Keeping the software open and running allows air to flow into the cages and the enclosure to maintain the selected temperature and light cycles. Thus, the entire system can operate with mice inside the cages for several days, even without measuring oxygen and CO_2.

1.3.6. Click on **Experiment**, then click on **Setup**, and the **Experiment Setup** window will open, where the parameters of each metabolic cage are defined.

1.3.7. Assign each mouse ID to the individual cage where the mouse is housed (**Figure 2C**).

1.3.8. Include the lean mass or total body weight for each mouse only if no differences in body weight are observed between groups.

NOTE: Obtaining raw values of energy expenditure facilitates ANCOVA analyses.

1.3.9. Set the airflow rate to the metabolic cage at 0.5-0.6 L/min.

1.3.10. On the Experiment Setup window, select the file saving path and name. Select the backup directory (**Figure 2D**).

1.3.11. Add a pre-weighted amount of food to the feeders, with the weight covering food intake for 1 day.

NOTE: If the cages have integrated scales, the food can be added directly, and the software will record it.

1.3.12. Add the water bottles. Check that the bottle is sealed correctly and does not leak.

1.3.13. 24 h after adding the food, weigh the food that is left on the cage.

NOTE: The grams of food added minus the grams of food left will measure food intake.

1.3.14. Start the oxygen, CO_2, and activity measurements (step 1.4.10) once the food intake values are the same as those of mice housed in regular cages.

NOTE: Here, the acclimation period (usually 2-3 days) is completed, and energy expenditure measurements can start.

1.4. Indirect calorimetry and activity measurements to assess energy expenditure

1.4.1. Measure the body weight, fat, and lean mass of all mice before starting the measurements.

NOTE: These are the body weight and lean mass values used to perform ANCOVA analyses.

1.4.2. Calibrate the CLAMS system's O_2 and CO_2 Zirconia-based detector (see **Table of Materials**) with the recommended oxygen concentration; always re-calibrate the detector before starting a new experiment.

1.4.3. Use a calibration gas of known composition (20.50% oxygen and 0.50% CO_2).

NOTE: Gas suppliers often refer to this gas as "Primary Standard Grade."

1.4.4. Turn on and ensure that the tank output pressure is at 5-10 psi.

1.4.5. Open the Calibration Utility software for calibrating and testing the gas sensors (**Figure 2E**). Click on **Experiment**, then **Calibrate**.

1.4.6. Press **Start**. Then, wait for the sensors to be tested and for the software to ask the user to turn the knobs of the gas sensor (**Figure 2F**) until the value of O_2 identity is 1 (**Figure 2G-H**). Click **Next** when the step is complete.

NOTE: If the Calibration Utility performs all current steps, the calibration will automatically proceed to the next step when the progress bar is filled.

1.4.7. Verify the calibration results when all steps have been completed, and the results are

presented.

1.4.8. Turn off the calibration gas.

1.4.9. Change food and add sufficient food for the mice for a 48-72 h period.

NOTE: Although cages could be opened during the energy expenditure measurements to monitor the body weight and change the food daily, mice can be stressed by these manipulations, and measurements are lost when the cages are opened. Thus, it is recommended to avoid any manipulation during the measurement period.

1.4.10. In the software, click on **Experiment** and then **Run** to start the oxygen, CO_2, and activity measurements (**Figure 3A**).

NOTE: The execution of the measurements can be tracked in real-time in a box located at the bottom left section of the software (red rectangle, **Figure 3B**). The red rectangle in **Figure 3B** shows that the system measures cage #1 at interval #3, namely the 3rd measurement. One measurement in one cage can take about 1 min. Thus, with 12 cages connected, oxygen consumption can be measured approximately every 12 min. Continuous measurements for a minimum of 48 h are recommended.

1.4.11. Stop the Experiment by clicking **Experiment** then **Stop** (**Figure 3C**).

1.4.12. Open the cages, weigh the mice and the food. Collect the feces to calculate the number of calories and lipids excreted over the 48-72 h measurements period.

NOTE: Feces can be stored at -20 °C for later analyses. These cages cannot be effectively used to collect urine.

1.4.13. Click on **Experiments**, then **Export,** and **Export all subjects** as a CSV file (**Figure 3D**).

NOTE: To facilitate ANCOVA analyses, it is essential to export raw Oxygen consumption (VO_2) and CO_2 production (VCO_2) values without being normalized by the body weight.

1.5. Data analysis and quality control

1.5.1. In the exported CSV sheet (**Figure 3D**) (from step 1.4.13), list all the raw values of the Oxygen consumption (VO_2) and CO_2 production (VCO_2) measured every 12 min in the 2-3 day period and include a timestamp, namely the hour and date that was measured.

NOTE: VO_2 and VCO_2 values will be automatically corrected if body weight or lean mass values are added.

1.5.2. In the exported CSV sheet, check that the raw values of the respiratory exchange ratio

(RER: VCO_2/VO_2) are automatically calculated by the software and listed.

NOTE: Values close to 1 show that the mouse primarily oxidizes carbohydrates, while values closer to 0.7 represent that the mouse is mainly oxidizing fat. RER above 1 can occur during anaerobic exercise, as the body expels more CO_2 to compensate for the acidosis caused by lactate. RER higher than 1 can indicate stress. The exported CSV file also contains the raw values from Energy expenditure (EE) or Heat production in calories per minute per mouse, measured every 12 min in the 2-3 days. Here, all the listed values include a timestamp.

1.5.3. As single EE values per mouse are needed for an ANCOVA, average the EE values recorded between 09:00-16:00 for the light (day) phase and 19:00-04:00 for the dark (night) phase per mouse and day.

NOTE: This can be done manually using Excel or Graph Pad. Selecting these two-time windows avoids averaging the intermediate, gradual, and unstable EE values associated with the light-dark phase transition.

1.5.4. For a 48 h period, calculate the average of the two daylight values and the two dark phase values per mouse using Excel or Graph Pad.

1.5.5. To quantify the total physical activity, use Excel or Graph Pad to sum the x, y, and z beam break counts measured in the metabolic cages and listed in the CSV file for each mouse.

NOTE: Cumulatively, one average value per parameter, mouse, and cycle will be obtained (being the average of 2 days).

1.5.6. Alternatively, represent the data showing each measurement value over time, which generates curves that illustrate the changes in EE during the transition from light to dark cycles.

NOTE: Please refer to the discussion section on how and when to perform ANCOVA analyses, and the different formulas used to calculate VO_2, VCO_2, and EE are provided in **Supplementary File 1**.

2. Measurement of the capacity of thermogenic adipocytes to expend energy

2.1. Set up the measurements and mouse treatments. See step 1 for details on the experimental preparations to monitor oxygen consumption, as thermogenic capacity in adipocytes is determined indirectly by oxygen consumption following steps 2.1.1-2.2.2.

NOTE: This protocol requires mouse anesthesia and acute treatment with the beta-3 receptor agonist CL-316,243 (see **Table of Materials**), giving a rapid assessment of BA thermogenic capacity.

2.1.1. Perform body composition analysis and weigh the mice. Turn on the CLAMS, set up the temperature at 30 °C (thermoneutrality), and wait for 2 h for the whole system to warm up.

2.1.2. Set up the rest of the assay conditions, including light, assign mouse ID to each cage and add bodyweight value of each mouse in the corresponding cage if no difference in body weight is observed between groups.

2.1.3. Calibrate the oxygen/CO_2 detector as in steps 1.4.2-1.4.7.

2.1.4. Start the Experiment in the software.

2.1.5. Open the metabolic cage and inject the mouse with pentobarbital (60-120 mg/kg), and place mice back in the metabolic cage.

NOTE: The dose of pentobarbital required to maintain mice asleep at thermoneutrality (30 °C) varies with the mouse strain and genotype. It is recommended to test different pentobarbital doses from 50 to 120 mg/kg and chose the one keeping the mouse anesthetized during 2-3 h at 30 °C. Efficient anesthesia is essential to remove the contribution of physical activity to energy expenditure.

2.1.6. To ensure anesthesia, observe mice after pentobarbital injection until they are completely asleep and their decreased oxygen consumption rates become steady.

2.1.7. Wait to obtain at least 3 stable consecutive oxygen consumption rates before injecting CL-316,243.

NOTE: While waiting for the stabilization of oxygen consumption, prepare the syringes with CL-316,243 for each mouse (1 mg/kg).

2.1.8. Open cage #1 and inject CL-316,243 subcutaneously immediately after one VO_2 and VCO_2 measurement occurred in cage #1. Return the mouse to cage #1 immediately after injection.

NOTE: Measurements are being indicated in real-time in the bottom-left section of the software (**Figure 3B**, red rectangle).

2.1.9. Wait for cage #2 to be measured (**Figure 3B**, red rectangle) and then proceed as in step 2.1.8 for cage #2.

NOTE: Injecting CL-316,243 immediately after a measurement allows maintaining the time constant between injections. For instance, if there are 12 mice/cages running, with measurements collected in individual cages sequentially and the collection lasting 55 s per cage, then you should inject one mouse every minute. With these injection rates, the first measurement will occur after 12 min in all 12 cages.

2.1.10. Continue the energy expenditure measurements until the energy expenditure values plateau for 5-6 consecutive measurements, usually 90-180 min after injection.

NOTE: Mice could wake up from the anesthesia during the experiments. These mice need to be removed from the analysis. Therefore, testing pentobarbital doses beforehand will increase the efficiency of the studies.

2.1.11. Stop the energy expenditure measurements, but keep mice at their cages at 30 °C, until they wake up.

2.1.12. After mice are fully awake, inspect the health of the mice and return them to their initial cages.

2.1.13. Export the data of each mouse as a CSV file using the equipment software, as described in section 1.4.13.

2.2. Data analysis

NOTE: The data analysis was performed by Excel or Graphpad

2.2.1. Plot the 3-5 consecutive values of VO_2, VCO_2, and EE that are stable and constant over time, as these are the values representing the metabolic rate when mice are fully anesthetized.

2.2.2. Then, plot the first and the following consecutive VO_2, VCO_2, and EE measurements obtained after injection.

NOTE: The absolute values of EE and the fold increase in EE induced by injection indicate BA thermogenic function[7].

REPRESENTATIVE RESULTS:

Figure 4 shows VO_2, VCO_2, Heat production/Energy expenditure (EE), Respiratory Exchange Ratio (RER), and X, Y, Z physical activity values obtained using the metabolic cages of the CLAMS system. The VO_2 and VCO_2 provided by the CLAMS system is the volume of gas (mL) per minute and can be already divided by the bodyweight or the lean mass values by entering these weight values in the CLAMS software before starting the measurements. However, bodyweight values must not be entered if differences in body weight between groups of mice are observed, as ANCOVA analysis is needed and the Oxymax software cannot perform these calculations. The energy expenditure (heat) is calculated in kcal/h using the Lusk equation. Mice are nocturnal and spend more energy during the night/dark period, which means that energy expenditure calculations need to be separated according to the light cycle. As expected, mice during the dark phase have higher O_2 consumption, CO_2 production, and thus higher EE, as shown in **Figure 4C**. Mice on a regular diet and in the fed state, with food ingestion occurring in the dark cycle, are characterized by RER values close to 1 (**Figure 4D**), meaning a preference to use carbohydrates. During the light cycle, when mice mostly sleep and thus fast, there is a shift to fat oxidation, with RER values being closer to 0.7. Accordingly, physical activity, measured as x,y,z laser beam break counts, increases during the dark phase and decreases during the light phase (**Figure 4E**).

We compared 16 week-old female mice fed a high-fat diet (8 weeks) to chow-fed mice, allowing the comparison of energy expenditure between groups of mice with differences in body weight. As expected, high-fat diet feeding increases fat mass without changing the lean mass (**Figure 5A-C**). High-fat diet-fed mice ate more Kcal/day, mainly due to higher caloric density per gram of food (**Figure 5D**). In addition, physical activity was similar between chow, and high-fat diet-fed mice, even during the dark period (**Figure 5E**). The lower values of RER show the preference of high-fat diet-fed mice to use fat as the primary substrate for oxidation, as expected with higher fat intake and muscle insulin resistance (**Figure 5F**). Oxygen consumption increases in high-fat diet-fed mice, but not CO_2 production (**Figure 5G-H**). The increase in oxygen consumption in high-fat diet-fed mice is accompanied by a significant increase in heat production/energy expenditure per mouse (**Figure 5I**). However, dividing energy expenditure by the lean mass of each mouse led to no differences in energy expenditure (**Figure 5J**), while dividing by total body weight showed a decrease in energy expenditure in high-fat diet-fed mice (**Figure 5K**). Cumulatively, these results indicate that dividing energy expenditure data by lean mass or total body weight can lead to opposite conclusions on the effects of high-fat diet feeding on energy expenditure. As suggested by multiple studies, the analysis of covariance (ANCOVA) allows determining whether differences in energy expenditure exist independently of the changes in body weight. To illustrate this point, an ANCOVA analysis was performed using the same data shown in **Figure 5A-K**, with energy expenditure being the dependent variable and body weight or lean mass as the covariates. While performing ANCOVA using total body weight as a covariate shows only a trend for high-fat diet-fed mice to have higher energy expenditure (**Figure 5L**), the high-fat diet-fed mice show a significant increase in energy expenditure when lean mass is used (**Figure 5M**). These data suggest that using total body weight to perform ANCOVA analyses could be underestimating energy expenditure[4]. The reasons can be that: (1) adipose tissue only contributes to ~5% of total energy expenditure and (2) the gain of fat mass induced by high-fat diet feeding results mainly from an expansion of triglyceride content in adipocytes, rather than from an increase in the number of oxidative thermogenic adipocytes.

Brown and beige adipocytes (BA) contribute to thermogenesis and consequently to energy expenditure in rodents. The contribution of BA to energy expenditure *in vivo* cannot be determined just by measuring whole-body oxygen consumption and calculating the BMR, as multiple tissues consume oxygen. The approach to determine BA thermogenic capacity *in vivo* involves anesthesia first, which is needed to limit oxygen consumption in all tissues. Then anesthesia is combined with a pharmacological approach to activate thermogenesis, mostly in thermogenic BA. As beta-3 adrenergic receptors are primarily expressed in adipose tissue, the beta-3 adrenergic agonist CL-316,243 can be used to activate BA thermogenic function. In addition, the anesthetized mice can be placed in a temperature-controlled enclosure at 30 °C, to prevent any uncontrolled sympathetic BA activation induced by ambient thermal stress. **Figure 6** shows mice fed a high-fat diet anesthetized with pentobarbital and placed in the metabolic cages at 30 °C, to record energy expenditure at the sub-standard metabolic rate (**Figure 6A-C,D**). This measurement was followed by CL-316,243 injection, which raised oxygen consumption, CO_2 production, and energy expenditure, as expected from BA activation (**Figure 6A-C**). A 2-3-fold increase in energy expenditure following beta-3 agonist treatment can be detected[7].

FIGURE LEGENDS:

Figure 1: The metabolic cages with the environmental enclosure and assembly of individual metabolic cages. (**A**) The metabolic cages in the environmental enclosure. (**B**) The enclosure can house 12 metabolic cages and allows controlling temperature and light. (**C**) Components of the metabolic cages before assembly. (**D**) Metabolic cages sealed with the lid.

Figure 2: Experimental setup and calibration of the oxygen sensor. (**A**) A screenshot of the Oxymax software controlling the metabolic cages, showing selection and opening of an "Experimental configuration" window to set. (**B**) Experimental properties, namely ambient light, and temperature. Then, the Experiment is configured using the (**C**) **"**Experimental Setup" window to assign a mouse ID, bodyweight, or lean mass to each cage, as well as the airflow rate for the 12 cages. (**D**) In the same "Experimental Setup" window, a file-saving path can be selected. (**E**) To calibrate the gas sensor, the user needs to turn the knob on the (**F**) gas detector to adjust the (**G-H**) O_2 identity to 1.

Figure 3: Start and stop of the measurements. (**A**) The Experiment is started by clicking on "Experiment," then "Run." (**B**) The users can see, real-time, which of the 12 cages is currently being measured (red rectangle), as well as a table with the measurements already collected. (**C**) The Experiment can be stopped by clicking on "Experiment," then "Stop." (**D**) The data can be exported to Excel by clicking on "File," then "Export," and then "Export all Subjects CSV."

Figure 4: Metabolic parameters obtained. (**A**) Oxygen consumption. (**B**) CO_2 production. (**C**) Energy Expenditure (EE) normalized to lean mass. (**D**) Respiratory exchange ratio (RER). (**E**) Physical Activity levels are calculated as the sum of X, Y, Z laser beam break counts. Data shows mean ± SEM. Student's *t*-test, **P < 0.01, ***P < 0.001. n = 7-8 female mice per group.

Figure 5: The ANCOVA analysis allows appropriate interpretation of changes in energy expenditure in obese mice. (**A-M**) Measurements in female mice fed either a chow or high-fat diet (HFD) for 8 weeks**.** (**A**) Bodyweight. (**B**) Fat mass. (**C**) Lean mass. (**D**) Food intake. Student's *t*-test, ***P < 0.001. (**E**) Physical activity was assessed with the metabolic cages as counts of laser beam breaks in X, Y, Z. (**F**) The respiratory coefficient ratio (RER). (**G**) Oxygen consumption (VO_2). (**H**) CO_2 production (VCO_2). (**I**) Energy expenditure (EE) was measured by indirect calorimetry. Energy expenditure was normalized to (**J**) Lean mass and (**K**) body weight. *P < 0.05 using Two-ANOVA. **P < 0.01, ***P < 0.001. (**L**) Covariate analysis (ANCOVA) of energy expenditure (EE) at night versus total body weight or (**M**) lean mass. Dashed lines represent the average body weight values modeled to determine VO_2 and EE in each group. *P < 0.05 using ANCOVA. n = 7-8 female mice per group. Data shows mean ± SEM.

Figure 6: The selective β3-agonist, CL-316,243 acutely increases energy expenditure in anesthetized mice at thermoneutrality. Female mice were anesthetized with pentobarbital (60 mg/kg) and placed in the metabolic cages set at 30 °C. Energy expenditure under anesthesia was recorded until 3 consecutive measurements showed the same values, reflecting complete

anesthesia. The mouse from cage #1 was injected with CL-316,243 (1 mg/kg) immediately after an oxygen consumption measurement. The same injection approach was used in the other cages to ensure that the same time passed between injection and the first measurement in all mice. (**A**) Oxygen consumption. (**B**) CO_2 production. (**C**) Energy expenditure. n = 4 female mice. Data shows mean ± SEM.

Supplementary File 1: Formulas used by Oxymax software in the CLAMS system to calculate oxygen consumption, CO_2 production, and energy expenditure.

DISCUSSION:

Indirect calorimetry has been used for years to assess whole-body energy expenditure[4]. This protocol described herein provides a straightforward method of measuring the basal metabolic rate and determining BA thermogenic capacity *in vivo* using metabolic cages.

The indirect calorimetry method described here confirms that dividing energy expenditure values by bodyweight values can be misleading. For example, it can conclude that energy expenditure is systematically lower in all mouse models with obesity. However, total energy expenditure can be higher in some mouse models of obesity, as in the case of an increase in food intake leading to obesity. Therefore, dividing energy expenditure by fat mass will always cause a misinterpretation of the process responsible for obesity in obese mice without primary defects in energy expenditure. In addition, dividing by lean mass is also inappropriate when changes in lean mass occur, as lean mass co-varies with energy expenditure, and energy expenditure can show a more significant decrease than any change in lean mass. This means that division of energy expenditure by body weight or lean mass can only be performed if no changes in body weight or body composition (i.e., lean mass and fat mass) are observed between the tested groups. As a consequence, the safest approach is to perform ANCOVA. This topic has been widely discussed in excellent articles, all of them concluding that an analysis of covariance (ANCOVA) is essential to compare energy expenditure between groups of mice with differences in total body weight or lean mass[4,5]. Here, SigmaPlot was used to perform ANCOVA analyses in-house, but many other advanced statistical analysis software can be used. The CalR website allows to upload data in one of their templates, but it might not always be possible depending on the experimental design[5]. Having statistical software to perform the ANCOVA "in-house" offers more flexibility on data analysis and presentation, but it is more time consuming[6].

Thermoneutrality for mice is around 30 °C, which suppresses the activity of thermogenic brown and beige adipocytes (BA), increasing heat production to combat low temperatures[1]. Ambient temperature (21 °C) is below thermoneutrality, meaning that BA thermogenesis will contribute to energy expenditure in mice housed at 21 °C. So, comparing energy expenditure at ambient temperature *vs*. thermoneutrality can be used to determine the contribution of BA to energy expenditure in a less invasive manner. However, this procedure requires the continuous use of the enclosure at 30 °C for 4 weeks, with thermoneutrality also causing differences in physical activity. In addition, thermoneutrality induces metabolic changes in other tissues, not just in BA. In a context where the main objective is to study changes in BA thermogenic capacity, the pharmacological approach described here has a list of advantages overexposing mice to

thermoneutrality over a long period:

Results are obtained in few hours, and the anesthesia suppresses the contribution of physical activity and other behavioral changes to energy expenditure. When assessing the effects of genetic manipulations in mice, metabolism might be changed in BA and other tissues. Thus, CL-316,243 treatment in anesthetized mice is the approach that can discern changes in BA activity with a higher dynamic range and specificity, with fewer confounders from energy expenditure stemming from other tissues. Alternatively, CL-316,243 can be injected in conscious mice as the system can measure physical activity. Therefore, if a change in physical activity occurs, it can be estimated and controlled[5]. In sum, while anesthesia can provide the highest dynamic range, measurements can be done without anesthesia if needed, as physical activity can be controlled.

When using the metabolic cages, caution must be taken regarding mice stress, and proper recovery is necessary. The social isolation of individual housing and the new environment of the metabolic cage stresses the mice, resulting in decreased food intake and weight loss. Thus, food intake and body weight need to be monitored every 24 h. Mice recover normal food intake 48-72 h after placing them into the metabolic cage. Thus, calibration and oxygen consumption measurements start when food intake is recovered. Despite the metabolic cages system being on, calibration and measures are not performed during this acclimation period, as by definition, the BMR must be obtained in a stress-free mouse. Avoiding measurements during this period increases detector lifespan by reducing the use and consumption of Drierite (which traps water to prevent oxygen detector damage). Newer and more expensive systems used home-cage-based measurements, which diminishes stress.

ANCOVA analyses

An ANCOVA (analysis of covariance) is needed when comparing energy expenditure in two groups of mice with differences in body weight[4]. The reason is that an increase in lean mass will increase energy expenditure. ANCOVA tests whether energy expenditure is statistically significantly changed between groups, independent of differences in body weight and lean mass. ANCOVA achieves that by determining whether energy expenditure differed if both groups had the same body weight or lean mass. However, to calculate the energy expenditure at the same body weight/lean mass using ANCOVA, the correlation between the covariate (bodyweight/lean mass) and the variable (energy expenditure) must be similar between groups. The similarity of this correlation is tested using Levene's test for equality of variance[5].

ANCOVA requires using more advanced statistical analysis software, such as SigmaPlot. Alternatively, different free websites can be used[5]. If ANCOVA shows that the effect observed between groups does not depend upon the value of the covariate (bodyweight/lean mass), the software will test whether the average of the variable (energy expenditure, VO_2, VCO_2) is different between the groups at a similar covariate (bodyweight/lean mass). The software will offer to make multiple comparisons with a suggested statistical test. If statistical significance is reached, it will confirm that the energy expenditure is significantly different between the two groups of mice at any given body weight value. The regression equation for the equal slopes model can be obtained from the analysis, which can be used in GraphPad or other graphical

software to generate a graph for publication[6].

Modifications and troubleshooting

The CLAMS system used in this protocol is constituted by small cages that are very different from the home cages that mice are used to, which include bedding. In addition, mice are social animals, and the need to house them individually, together with a new cage without bedding, causes initial stress to the mice. Thus, an acclimation of at least 2 days is necessary to allow mice to adapt to their new environments and mitigate stress. Usually, food intake comes back to what was recorded in their home cages on the third day. This acclimation period is unnecessary to assess BA capacity to expend energy, as it is performed in anesthetized mice.

Pentobarbital is a short-acting barbiturate that can be used as a sedative or anesthetic agent, but it is also used for euthanasia at higher doses. For an unknown reason, it was sometimes noticed that the efficacy of pentobarbital at 30 °C is different than at ambient temperature. Therefore, it is advised to test different pentobarbital doses in the mouse model at thermoneutrality. The main adverse effects of pentobarbital include respiratory depression and cardiovascular effects, such as reduced blood pressure, stroke volume, and hypotension[8].

Limitations

Beta3-adrenergic receptors are expressed in adipose tissue and detectable in the myocardium, retina, gallbladder, brain, urinary bladder, and blood vessels[9]. As such, CL-316,243 can potentially increase energy expenditure in these other tissues where the receptor is expressed. However, it was demonstrated that most of the energy expenditure induced by CL-316,243 in control mice is UCP-1 dependent, a BA-specific protein[10,11]. It needs to be taken into consideration that some genetic modifications may exacerbate the actions of CL-316,243 in other tissues. In addition, the fraction of UCP1 independent respiration can still be driven by ATP-consuming futile cycles described in activated adipose tissue.

ACKNOWLEDGMENTS:

ML is funded by the Department of Medicine at UCLA, pilot grants from P30 DK 41301 (UCLA:DDRC NIH) and P30 DK063491 (UCSD-UCLA DERC).

DISCLOSURES:

The authors declare no conflict of interest to this protocol paper. M.L. is a co-founder and consultant for Enspire Bio LLC.

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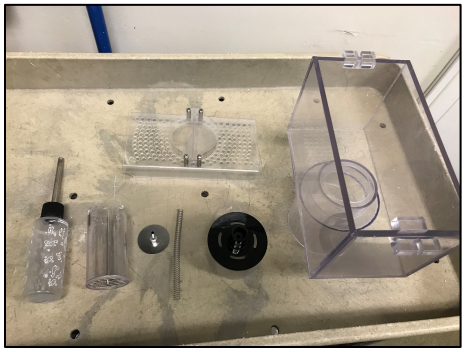
Figure 1

A

B

C



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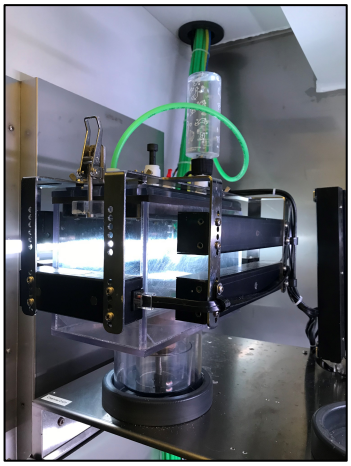


Figure 2

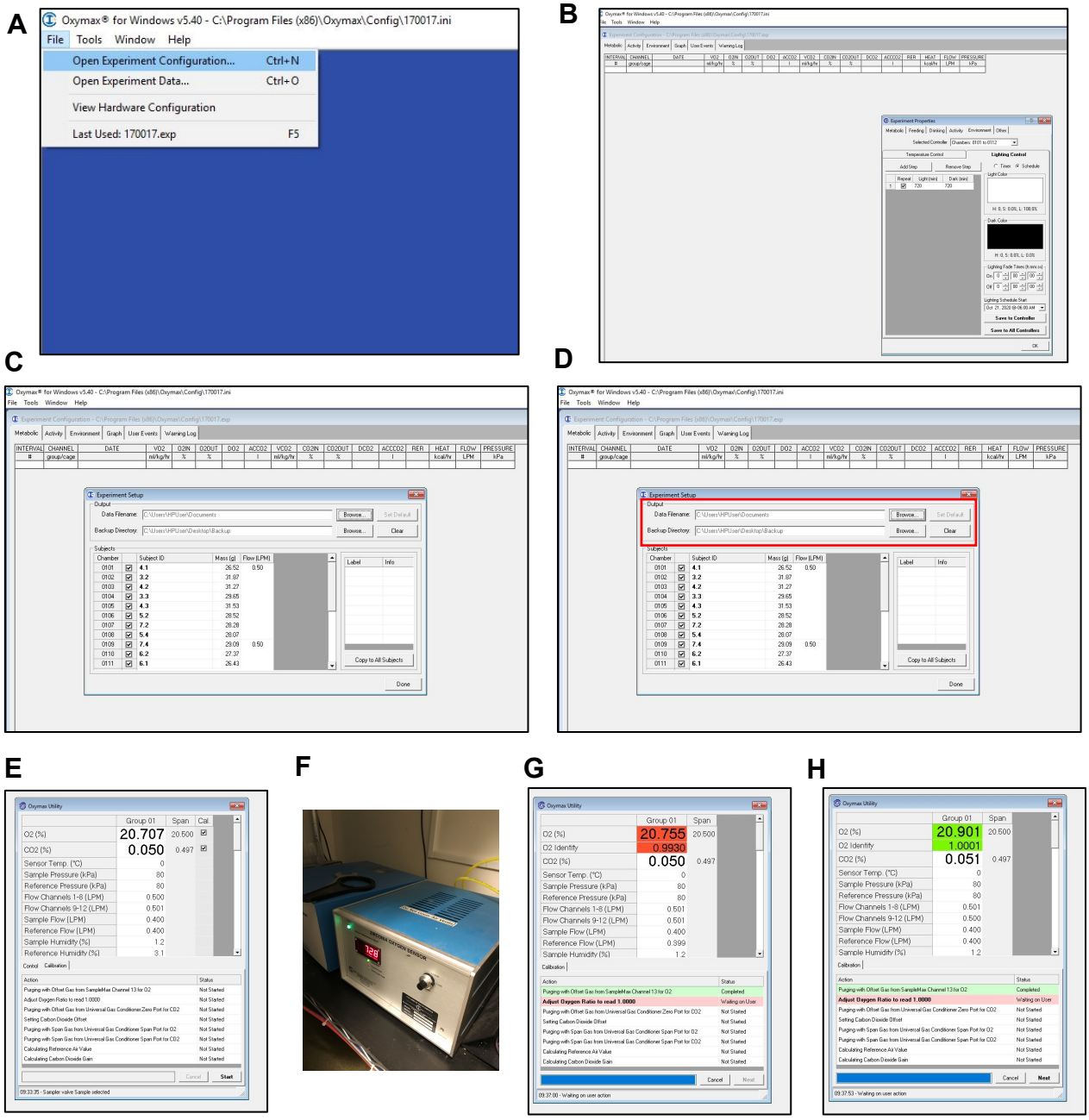
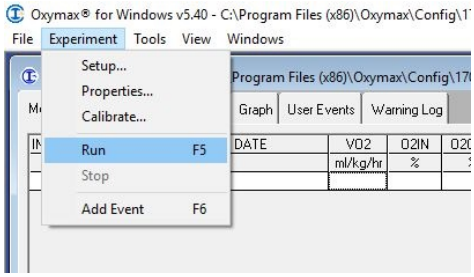


Figure 3

A



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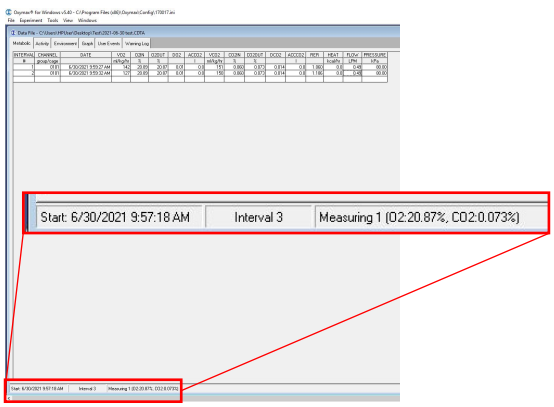

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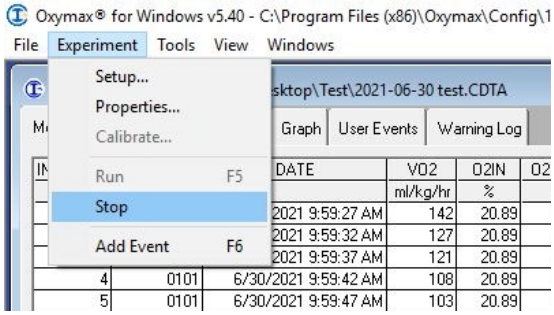

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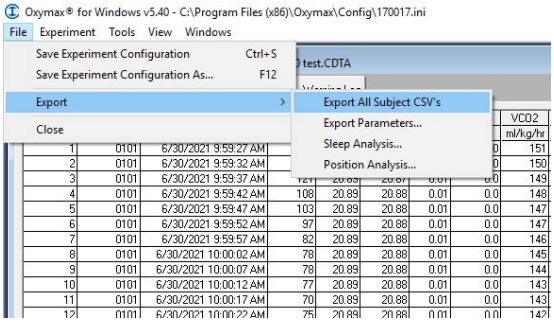


Figure 4

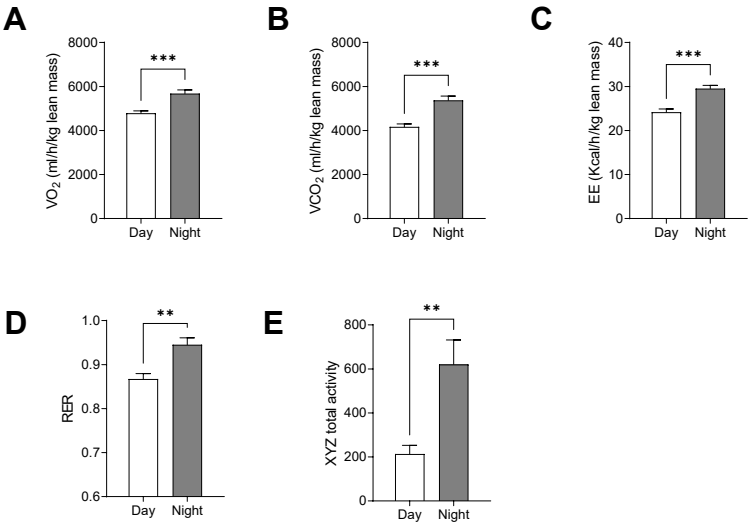


Figure 5

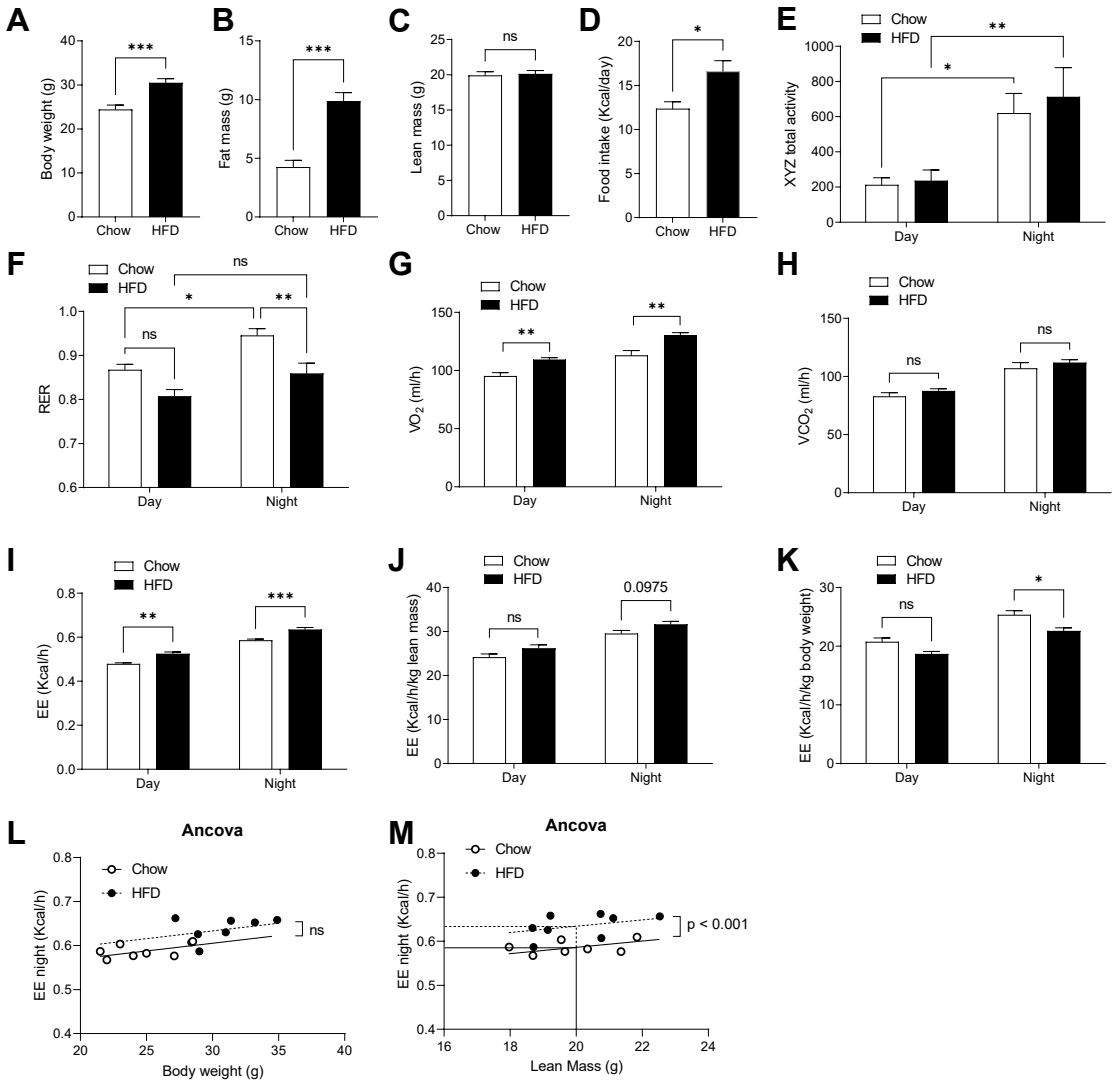
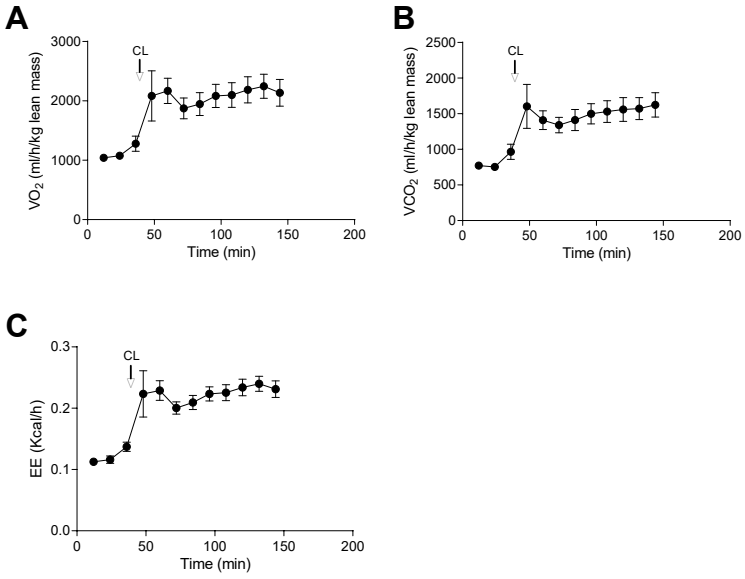


Figure 6



We thank the reviewer for reviewing our manuscript and appreciated the time they dedicated evaluating our work. Here are the responses to the editorial and reviewer's comments.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread our manuscript to correct spelling and grammar issues.

2. Please ensure that abbreviations are defined at first usage.

The abbreviations were defined at first use

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We deleted the personal pronouns.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials (including reagents, instruments, software, etc.). Please sort the Materials Table alphabetically by the name of the material.

We have added the Table of Materials in the manuscript and sorted alphabetically. We removed commercial language (i.e. CLAMS) and substitute it by Metabolic cages.

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

The numbering was corrected accordingly.

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

We have ensured that all steps have a maximum of 4 sentences (2-3 lines and actions).

7. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

We have moved the ANCOVA and description of the formulas to the Discussion section.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

We made sure that all the steps were written in the imperative tense. We also added a note section when needed.

9. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We added enough details in each step to support the actions seen in the video.

10. Please add more details to your protocol steps:
Step 1: Please specify the age/gender/strain of the mouse used.

We added the age/gender/strain of the mouse used.

11. The steps that include mathematical calculations such as data analysis, etc., please include them in the Discussion sections as the Protocol should consist of only action steps in the imperative tense.

We added the formulas and calculations in the discussion section and only included the specific actions needed for data analysis in the imperative tense.

12. In the software, please ensure that all button clicks and user inputs are provided throughout. Also, please ensure that the button clicks are bolded.

We made sure all the button clicks are bolded.

13. Please include one-line space between each protocol step and then highlight in yellow up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted the steps that are essential for the video. We excluded the steps that involved handling live mice.

14. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next and is in line with the Title of the manuscript. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in the imperative tense.

We have highlighted the steps as such.

15. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol. We provided that, highlighting the importance of ANCOVA analyses and body weight measurements.
b) Any modifications and troubleshooting of the technique. These have been included as imperative actions in the protocol and corresponding notes.
c) Any limitations of the technique. We added a limitations paragraph.
d) The significance with respect to existing methods. In the limitations paragraph, we compare it to other methods.
e) Any future applications of the technique.

In our discussion, we cover in detail the points mentioned above.

16. Please submit each figure individually as a vector image file to ensure high resolution throughout production.

All figures were submitted as .pdf.

17. In Figure 4/Figure 5, please note that VCO2 and VO2, the '2' should be subscripted.

Corrected

18. In Figure 5/6, please note that VCO2 and VO2, the '2' should be subscripted. Also, please note that hour should be represented as h and not hr.

Corrected

19. Please spell out the journal titles in the References.

The complete journal titles are now shown in the references.

Reviewers' comments:

Reviewer #1:

In this manuscript, Shum and colleagues detailed the guidelines for the evaluation of basal metabolic rate and BAT thermogenic capacity in mice using indirect calorimetry (oxygen consumption). These are important methods performed in most studies investigating metabolism and therefore they are of extreme relevance. Having a guideline such this may help those that are new to the field, and create a standard procedure that may minimize discrepancies between studies. One major issue that raises heated discussion regarding indirect calorimetry is how to express rates of oxygen consumption. In the literature, one may see rates of oxygen consumption expressed by mouse, grams of Kg of body weight, lean mass, body weight raised to 0.75, among others. These discrepancies have a major impact in the interpretation of findings. The use o ANCOVA suggested by authors and many others is an excellent strategy to discriminate the impact of body weight on rates of oxygen consumption. Regarding the evaluation of UCP1 mediated thermogenic capacity using CL, some groups, including mine, have performed those assays in awake instead of anesthetized mice. In my own experience locomotor activity is not a major confounding factor in this assay (sum of movement in x, y and z) and using awake freely moving mice may avoid the hypothermia associated with the use of anesthetics. Regarding the latter,

some groups had to increase environmental temperature to 32C to minimize the impact of hypothermia on the analysis. Perhaps authors could mention that the procedure may be executed in awake freely moving mice, considering that locomotor activity is concomitantly evaluated along oxygen consumption.

We thank the reviewer for the comments. We added in the discussion that, in some studies, locomotor activity was not a major confounding factor and thus anesthesia can be skipped as necessary, but with the risk of decreasing dynamic range. In addition, we further discuss that physical activity is measured in the assay and thus can be controlled if needed.

One final suggestion is to use the term UCP-1 mediated thermogenic capacity in mice in the title to account for the contribution of beige adipocytes.

This is a fantastic suggestion, as indeed the increase in oxygen consumption induced by CL-3164132 is mostly dependent on UCP1 expression. However, brown and beige adipocyte in response to adrenergic stimuli also enhance futile cycles consuming ATP, which can explain the remaining actions of CL-3164132 treatment in UCP1 KO mice. For this reason, we added “brown/beige adipocytes” in the title to account for the contribution of beige adipocytes, rather than just UCP1, to avoid the exclusion of these other processes that are UCP1-independent.

Reviewer #2:
Manuscript Summary:
The manuscript entitled " Determining basal energy expenditure and the capacity of brown adipocytes to expend energy in obese mice" by Michael Shum et al. described two basic methods to measure basal energy expenditure and thermogenic capacity of brown adipocytes in obese mice using metabolic cages. The comprehensive protocols provide strong support for researchers to study thermogenesis.

We thank the reviewer for the positive comments and the time dedicated to review our protocol.

Major Concerns:
In the Discussion part line 459, the authors claimed 'dividing energy expenditure values by body weight values can be misleading' and gave an explanation, however, it would be better to divide energy expenditure by lean mass values. Authors should discuss more details about why using body mass rather than lean mass.

We thank the reviewer for the comments. We have clarified that division per total body weight and even division per lean mass is inappropriate when there are changes in body weight or lean mass between groups. The reason is that body weight and lean mass co-vary together with energy expenditure, and energy expenditure can change independently from differences in lean mass.

Minor Concerns:
The manuscript still needs some polish, especially the tense.

We apologize for the tense. We revised our manuscript accordingly.

Formulas to calculate oxygen consumption, CO_2 production, and energy expenditure

(1) Oxygen consumption ($VO_{2,\ Subject} = V_i\ O_{2i} - V_o\ O_{2o}$)

(2) CO_2 production ($VCO_{2,\ Subject} = V_oCO_{2o} – V_iCO_{2i}$).

where V_i = volume input airflow (mL),
V_o = volume output airflow (mL),
O_{2i} = the input O_2 concentration,
O_{2o} = the output O_2,
CO_{2i} = the input CO_2 concentration,
CO_{2o} = the output CO_2.
$VO_{2,\ Subject,}$ and $VCO_{2,\ Subject}$ values are automatically corrected by the software for body weight or lean mass in Kg.

(3) Energy expenditure (EE) or Heat production in calories per minute per mouse are obtained by multiplying CV * $VO_{2\ Subject.}$

CV = calorific value is derived from Graham Lusk's empirical table, to generate the following formula:

CV = 3.815 + 1.232 * RER (respiratory exchange ratio).