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The 24-hour respiratory quotient predicts energy intake and changes in body mass

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Longo KA, Charoenthongtrakul S, Giuliana DJ, Govek EK, McDonagh T, DiStefano PS, Geddes BJ. The 24-hour respiratory quotient predicts energy intake and changes in body mass. *Am J Physiol Regul Integr Comp Physiol* 298: R747–R754, 2010. First published December 16, 2009; doi:10.1152/ajpregu.00476.2009.—To define the relationship between the respiratory quotient (RQ) and energy intake (EI) and to determine the impact of spontaneous locomotor activity (LMA) in the development of diet-induced obesity (DIO), we fed C57BL/6 mice a high-fat diet (HFD) for either 4 days or 17 wk and analyzed them using indirect calorimetry. Importantly, changes in body mass during calorimetry (ΔM_b) significantly covaried with RQ and EI; adjusting the data for ΔM_b permitted an analysis of the energy-balanced state. The 24-h RQ strongly predicted 24-h EI, and the slope of this relationship was diet dependent (HFD or chow) but independent of the HFD feeding period. Early-stage DIO was characterized by dark-period hyperphagia and fat storage, offset by greater light-period lipid oxidation; later stage DIO mice had a milder hyperphagia and lower substrate flexibility. Consequently, whereas 24-h RQ equaled the food quotient of the HFD in both early- and late-stage DIO, the range of RQ values was negatively correlated with, and mostly explained by, 24-h EI only in late-stage DIO. Lean and early-stage DIO mice had similar LMA values that were reduced in late-stage DIO. However, LMA significantly explained variance in total energy expenditure (EE) in only early-stage DIO mice. This indicated that the link between LMA and EE was a transient adaptive response to early DIO, whereas the later loss of LMA did not explain body weight gain in C57BL/6 DIO mice.

diet-induced obesity; energy expenditure; substrate flexibility; indirect calorimetry

WHEN FED A HIGH-FAT DIET (HFD), the C57BL/6 mouse develops diet-induced obesity (DIO) and insulin resistance (1, 4–5, 8, 12, 31). The causes underlying this susceptibility are multifactorial, likely involving complex interactions among genetic, epigenetic, and environmental factors. Genetic mutations, or the altered expression of genes involved in adipocyte function and energy expenditure, may predispose these mice to DIO (16, 34). Hyperphagia, which is an initial transient response to HFDs in C57BL/6 mice (23), may factor less in their development of DIO than their greater efficiency at storing energy as fat in adipose tissue (6, 16, 25, 35). In addition, the progressive leptin resistance that develops as a consequence of DIO promotes food intake and reduces energy expenditure, resulting in further weight gain (18, 24, 36). A topic of continuing interest has been the role of locomotor activity (LMA) in the development of DIO. Although one report indicated that LMA was not different between lean and DIO mice and was not a factor in the diet-induced weight gain (6), another study showed that LMA was rapidly reduced in mice fed a HFD and accounted for the loss of a significant portion of daily energy expenditure and weight gain (4). Thus our understanding of the fundaments of energy imbalance in this widely used animal model is an ongoing process.

Indirect calorimetry is commonly used in small animal energy balance studies, and modern systems can simultaneously measure multiple parameters such as oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$), food intake, and LMA (2). The ratio of $\dot{V}CO_2$ and $\dot{V}O_2$, otherwise known as the respiratory quotient (RQ), can be used to estimate fuel utilization and to calculate energy expenditure (EE) (21, 38). What is often missing from calorimetry study reports is the change in body mass during the measurement period (ΔM_b); energy balance is tacitly assumed. However, the failure to account for ΔM_b can potentially lead to interpretive errors, since the measurements may be either fully or partially attributable to energy imbalances rather than the main effect being tested. In practical terms, ensuring that all of the animals in a particular experiment remained in energy balance would be unfeasible; however, this could be achieved statistically by adjusting the data for ΔM_b by using least-squares linear regression, potentially improving the power of any comparison.

With this in mind, we used indirect calorimetry to examine energy balance in DIO C57BL/6 mice and to determine the effect of short- and long-term consumption of a HFD on the relationship between the RQ and energy intake (EI), as well as the influence of LMA on EE and the susceptibility to DIO. Importantly, we compared the estimates or residuals of these variables after adjusting for the ΔM_b that occurred during calorimetry.

METHODS

Animals and diets. The Elixir Pharmaceuticals Institutional Animal Care and Use Committee approved all protocols for the experimental use of animals, in accordance with National Institutes of Health guidelines. Individually housed C57BL/6N mice (Taconic, Germantown, NY) were kept in ventilated racks (Thoren, Hazelton, PA) in a controlled environment: 72°F, ~40% humidity, and 12-h light and dark periods (lights on/off at 6:00 AM/6:00 PM). Mice were weaned at 3 wk of age on chow; at 6 wk one group continued on chow (lean), whereas another was switched to a HFD (LTDIO) for 17 wk. A third group of mice given chow for 17 wk was switched to HFD for the 4 days of calorimetry (STDIO). During calorimetry, mice received mashed forms of the diets. The food quotient (FQ), defined as the ideal diet-specific ratio of $\dot{V}CO_2$ to $\dot{V}O_2$ (3), was calculated for each diet using the following equation (20): (0.835 × %protein) + (1.0 × %carbohydrate) + (0.71 × %fat). The chow diet (Lab Diet 5001; Purina; St. Louis, MO) contained 28.5% energy from protein, 58% energy from carbohydrate, and 13.5% energy from fat, with a calcu-

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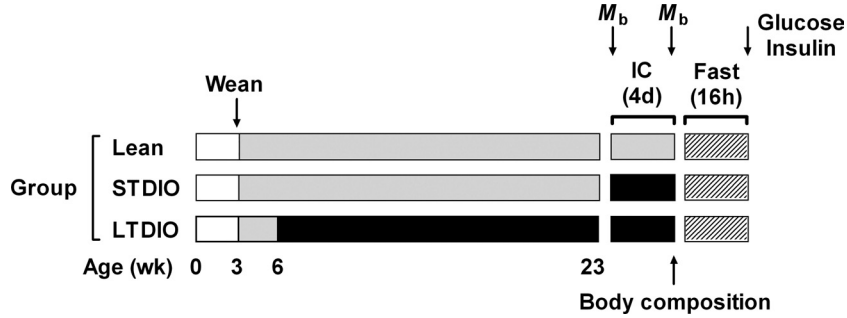

Fig. 1. Design and timeline of the experiment. C57BL/6 mice were given a chow diet (shaded bars) for 17 wk (lean group) or a high-fat diet (HFD; solid bars) for 17 wk (LTDIO group) or a chow diet for 17 wk followed by HFD for 4 days (STDIO group). Indirect calorimetry (IC) was performed for 4 days, followed by a 16-h fast (hatched bars); body mass (M_b), body composition, blood glucose concentration, and plasma insulin level were determined at the times indicated (arrows).

lated FQ of 0.917 and an energy equivalent of 3.44 kcal/g. (A nutrient-matched "low-fat" diet was not used due to concerns about its high sucrose content and its potential effects on insulin resistance.) The HFD (D12492; Research Diets, Rahway, NJ) contained 20% energy from protein, 20% energy from carbohydrate, and 60% energy from fat, with a calculated FQ of 0.793 and an energy equivalent of 5.24 kcal/g. Alternate FQ estimates were made for Lab Diet 5001 (~0.855) and D12492 (~0.766) with the use of this and other data sets by linear regression analysis of ΔM_b (*x*-axis) and 24-h RQ (*y*-axis); the *y*-intercepts were the 24-h RQ when ΔM_b equaled zero, or theoretical FQ.

Indirect calorimetry. Food intake, $\dot{V}_{CO_2}$, $\dot{V}_{O_2}$, and *x*-axis LMA were recorded using a 16-chamber indirect calorimetry system (Oxymax; Columbus Instruments, Columbus, OH), which was calibrated using a defined mixture of O_2 and CO_2. Measurements were taken from mice every 14.1 min for 96 h. The M_b of each mouse was recorded immediately before and after chamber occupancy, and food waste was accounted for. LMA was defined as the total number of *x*-axis infrared beam breaks. [Consecutive ambulatory beam breaks were highly colinear with total beam breaks ($r = 0.977$, $R^2 = 0.955$, $P < 10^{-10}$); thus either measurement conveyed similar information.] RQ was calculated as the quotient of $\dot{V}_{CO_2}$ and $\dot{V}_{O_2}$, and EE (kcal/day) was calculated with the equation $[3.815 + (1.232 \times RQ)] \times \dot{V}_{O_2}$, with $\dot{V}_{O_2}$ defined as liters of O_2 per day (21, 38). For the measurement of RQ range (ΔRQ), relative cumulative frequency (RCF) curve analysis was used (29). The ΔRQ was defined as the inverse Hill slope of the RCF curve, normalized to the lean group (ΔRQ = 1).

Body composition analysis. Lean tissue mass and fat mass (g) were determined in mice at the completion of calorimetry using a Bruker Minispec model LF50 (Bruker Optics, Billerica, MA).

Blood and plasma analyses. After body composition analysis was completed, the mice were fasted overnight for 16 h. The next morning, blood glucose was measured via tail nick using a glucometer (Ascencia Elite; Bayer, Indianapolis, IN), and plasma insulin was measured using a homogeneous time-resolved fluorescence immunoassay (Cisbio-US, Bedford, MA). Insulin resistance was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR) (22): [fasting blood glucose (mM) × fasting plasma insulin (mU/l)]/22.5. Intra- and inter-assay coefficients of variance (CVs) were 4.3 and 5.4% (glucose) and 8.3 and 10.5% (insulin), respectively.

Statistics. Analysis of variance and Tukey's honestly significant difference test were used to assess group means, which were considered significantly different at $P < 0.05$. Statistical trends were defined as $0.05 \leq P < 0.1$. Relationships between independent (*x*) and dependent (*y*) variables were assessed using linear least-squares regression, and in some cases regression was performed on the residuals of a dependent variable after adjusting for one or more independent variables. Correlations were evaluated with the Pearson coefficient (*r*), with the R^2 indicating the fraction of variance in *y* described by *x*, and significance was determined using the Bonferroni probability test. Statistical analyses conformed to the guidelines of the American Physiological Society (10) and were performed with SYSTAT version 12 (Systat Software, Chicago, IL) and GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego CA).

RESULTS

Early and long-term physical and biochemical changes in response to HFD. Figure 1 depicts the experimental design and timing of measurements. In brief, two groups of 6-wk-old mice were fed for 17 wk either chow (lean group) or HFD (long-term DIO; LTDIO group) and then analyzed with indirect calorimetry; a third chow-fed group was switched to the HFD for the 4 days of calorimetry (short-term DIO; STDIO group). Immediately before calorimetry, a significant albeit predictable diet-induced increase in M_b (43%) was noted in the LTDIO mice relative to the lean mice (Table 1). The lean and LTDIO groups had a negative energy imbalance during calorimetry, losing 6.2 and 2.7% of their M_b, respectively; in contrast, when shifted to the HFD, the STDIO mice had an 8.1% increase in M_b (Table 1). Several effects observed in the STDIO group compared with the lean group, including greater fasting glucose, fasting insulin, and HOMA-IR, were completely explained after adjusting for ΔM_b (Table 1). Therefore, the acute changes in fat mass due to shifts in energy balance, and not the switch in diet macronutrients per se, accounted for these differences.

Table 1. *Physical and biochemical parameters from lean, LTDIO, or STDIO C57BL/6 mice*

Variable	Treatment Groups		
	Lean	STDIO	LTDIO
M_b (precalorimetry), g	34.29 ± 2.45	31.8 ± 3.07*	47.15 ± 2.43†‡
M_b (postcalorimetry), g	32.18 ± 2.09	34.36 ± 3.34*	45.86 ± 2.38†‡
ΔM_b (post − pre), g	−2.12 ± 0.98	2.57 ± 0.81*	−1.29 ± 0.88†‡
Unadjusted			
Fat mass, g	6.85 ± 1.79	8.67 ± 2.49*	17.36 ± 1.46†‡
Fat-free mass, g	16.79 ± 0.83	17.29 ± 1.27	20.86 ± 1.06†‡
Glucose, mg/dl	53 ± 6	76 ± 12*	87 ± 11†‡
Insulin, ng/ml	0.81 ± 0.31	0.7 ± 0.28	2.75 ± 0.94†‡
HOMA-IR§	2.48 ± 1	3.23 ± 1.74	13.99 ± 5.42†‡
Adjusted for ΔM_b			
Fat mass, g	7.19 ± 0.56	8.22 ± 0.75	17.64 ± 0.43†‡
Fat-free mass, g	16.85 ± 0.31	17.21 ± 0.41	20.93 ± 0.24†‡
Glucose, mg/dl	60 ± 3	66 ± 4	91 ± 2†‡
Insulin, ng/ml	0.76 ± 0.17	0.76 ± 0.22	2.77 ± 0.13†‡
HOMA-IR§	2.72 ± 0.94	2.88 ± 1.26	14.31 ± 0.72†‡

Parameters were measured in C57BL/6 mice fed a chow diet for 17 wk (lean), a long-term high-fat diet (HFD) for 17 wk (LTDIO), or a short-term HFD for 4 days (STDIO); $n = 32$ mice/group. Unadjusted values are means ± SD; adjusted values are means ± SE. Tukey's post hoc statistical comparisons: *$P < 0.001$, lean vs. STDIO; †$P < 0.001$, STDIO vs. LTDIO; ‡$P < 0.001$, lean vs. LTDIO. §Units for homeostasis model assessment of insulin resistance (HOMA-IR): glucose (mM) × insulin (mU/l)/22.5.

Table 2. *Metabolic parameters from lean, LTDIO, or STDIO C57BL/6 mice*

Variable	Treatment Groups		
	Lean	STDIO	LTDIO
Unadjusted			
EI_{24h}, kcal/24 h	9.81 ± 1.93	19.94 ± 1.61*	13.28 ± 2.42†‡
EI_{light}, kcal/12 h	2.79 ± 0.73	6.4 ± 1.21*	4.03 ± 1.37†‡
EI_{dark}, kcal/12 h	7.02 ± 1.49	13.54 ± 1.17*	9.25 ± 1.32†‡
EE, kcal/24 h	15.06 ± 1.06	17.83 ± 1.28*	18.98 ± 1.86†‡
LMA, beam break/24 h	85,355 ± 22,154	77,259 ± 23,829	59,018 ± 12,588†‡
RQ, $(\dot{V}_{CO_2}/\dot{V}_{O_2})$/24 h	0.820 ± 0.019	0.790 ± 0.01*	0.757 ± 0.009†‡
ΔRQ§	1 ± 0.165	0.637 ± 0.07*	0.495 ± 0.096†‡
Adjusted for ΔM_b			
EI_{24h}, kcal/24 h	11.95 ± 0.29	16.33 ± 0.39*	14.28 ± 0.22†‡
EI_{light}, kcal/12 h	3.85 ± 0.22	4.75 ± 0.29*	4.4 ± 0.17‡
EI_{dark}, kcal/12 h	8.11 ± 0.26	11.58 ± 0.34*	9.88 ± 0.2†‡
EE, kcal/24 h	14.83 ± 0.42	18.19 ± 0.56*	18.91 ± 0.32‡
LMA, beam break/24 h	81,090 ± 4,896	81,600 ± 6,630	57,324 ± 3,774†‡
RQ, $(\dot{V}_{CO_2}/\dot{V}_{O_2})$/24 h	0.855 ± 0.005	0.768 ± 0.004*	0.765 ± 0.002‡
ΔRQ§	1.133 ± 0.065	0.738 ± 0.03*	0.46 ± 0.03†‡

Metabolic parameters were measured in lean, LTDIO, or STDIO C57BL/6 mice; $n = 32$ mice/group. Energy intake is given for the 24-h and 12:12-h light-dark periods, as indicated. EE, energy expenditure; LMA, locomotor activity; RQ, respiratory quotient. Unadjusted values are means ± SD; adjusted values are means ± SE. Tukey's post hoc statistical comparisons: *$P < 0.001$, lean vs. STDIO; †$P < 0.001$, STDIO vs. LTDIO; ‡$P < 0.001$, lean vs. LTDIO. §Inverse Hill slope, normalized to lean group value.

Early and long-term changes in EI and EE in response to HFD. The STDIO mice had significant dark-phase hyperphagia relative to the LTDIO group; this was also evident in the predicted energy-balanced state (when $\Delta M_b = 0$) (Table 1). Compared with the lean group, 24-h EE was greater in both DIO groups (Table 2) and was correlated with fat-free mass (data not shown). The rapid increase in 24-h EE in the STDIO group was likely due to its greater EI and the associated thermic effect of food, since their fat-free mass was equivalent to that of the lean group (Table 1) (11, 32).

Relationship between LMA and EE in short- and long-term DIO. Locomotor activity was similar in the lean and STDIO mice but lower in the LTDIO mice (Table 2). However, 24-h LMA and EE (kcal/24 h) were not correlated in any of the groups (Table 3). The significant group differences in M_b and energy imbalance (i.e., ΔM_b) prompted an examination of their effects on EE and LMA; several significant effects and trends were found in the DIO groups (Table 3). When residual analysis was used to remove the contributions of M_b and energy imbalance from EE and LMA (2, 33), LMA significantly explained 22% of the variance in EE in the STDIO group but <1% of the variance in EE in the lean or LTDIO groups (Table 3). Therefore, although LMA was reduced after several weeks of the HFD, it was a minor component of EE and could not explain the long-term diet-induced weight gain. However, short-term HFD consumption induced a significant relationship between LMA and EE.

Changes in RQ during early and late-stage DIO. During calorimetry, the lean group exhibited a diurnal variation in its RQ (Fig. 2*A*). Comparatively, the LTDIO mice had a significantly lower 24-h RQ value that approached the FQ value of the HFD, and the peak-to-peak amplitude of RQ was reduced (Fig. 2*A*). Notably, the RQ values in the lean and LTDIO groups trended higher over time as their food intake normalized during cage acclimation (data not shown). The RQ values of the STDIO mice gradually converged with those of the LTDIO group, as the STDIO mice adjusted to the fuel mix of the HFD (Fig. 2*A*).

The unadjusted 24-h RQ value defined the direction of energy imbalance. In the STDIO group, the 24-h RQ value exceeded the FQ value of the HFD by 0.024 units (Table 2); these mice gained M_b during calorimetry. The lean and LTDIO groups each had 24-h RQ values below the FQ values of their respective diets and lost M_b during calorimetry (Table 2). A comparison of the unadjusted and ΔM_b-adjusted 24-h EI data for the STDIO mice indicated that an energy excess of 22%

Table 3. *Relationships between LMA and 24-h EE, including the influence of M_b and the change in M_b during calorimetry*

Variable		Treatment Groups								
		Lean			STDIO			LTDIO		
x	*y*	*r*	R^2	*P* value	*r*	R^2	*P* value	*r*	R^2	*P* value
LMA	EE	0.022	0.000	0.906	0.229	0.052	0.215	0.107	0.012	0.572
M_b	EE	0.062	0.004	0.742	0.234	0.055	0.206	0.468	0.219	0.009
M_b	LMA	−0.018	0.000	0.922	−0.446	0.199	0.012	0.311	0.097	0.095
ΔM_b	EE	−0.262	0.068	0.155	0.32	0.102	0.08	−0.225	0.051	0.231
ΔM_b	LMA	−0.018	0.000	0.924	−0.341	0.116	0.061	−0.136	0.019	0.473
LMA*	EE*	0.017	0.001	0.928	0.47	0.221	0.008	−0.076	0.006	0.691

Relationships were determined between LMA (beam breaks/24 h) and 24-h EE (kcal/24 h), including the influence of body mass (M_b, g) and the change in body mass during calorimetry (ΔM_b, g/4 days), in lean, STDIO, and LTDIO mice. Pearson's correlation coefficient (*r*), goodness of fit (R^2), and significance (*P* value) are given for each regression. *Adjusted for M_b and ΔM_b.

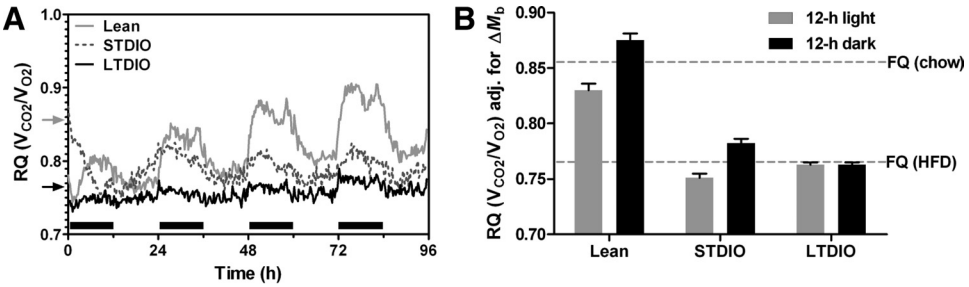

Fig. 2. *A*: respiratory quotient (RQ) over 4 days in lean, LTDIO, or STDIO mice. Solid horizontal bars indicate the 12-h dark periods. $\dot{V}CO_2$, CO_2 uptake; $\dot{V}O_2$, O_2 consumption. *B*: average periodic RQ from the same mice, adjusted for changes in body mass (ΔM_b). Data are means ± SE during the light and dark periods. Arrows or dashed horizontal lines mark the locations of the food quotients (FQ) for the chow (0.855) and HFD (0.766).

(3.61 kcal/day) promoted their gain in M_b during calorimetry (Table 2). [Using regression analysis, the FQ values of the chow and HFD were estimated to be 0.855 and 0.766, respectively; these values provided a more accurate context for the present data but differed from those calculated using an equation, particularly for the chow diet (20). The equation method for estimating FQ can be prone to error, especially for diets with poorly defined macronutrients or for substrate-specific FQ values that deviate significantly from the equation constants (19–20).]

The STDIO mice rapidly adapted to the HFD; the 24-h RQ value (adjusted for ΔM_b) was similar between the STDIO and LTDIO groups and close to the FQ value of the HFD (Table 2). The STDIO mice were predicted to maintain energy balance through greater energy storage in the dark period (RQ > FQ), offset by greater oxidation of stored lipid during the light period (RQ < FQ) (Fig. 2*B*). In contrast, the LTDIO mice had a loss of phasic substrate flexibility, with average periodic RQ values ≈ FQ (Fig. 2*B*); these mice were in a greater equilibrium with their diet macronutrients and caloric intake, had smaller shifts in fat storage and oxidation, and therefore maintained adipose tissue mass at a more constant level.

The RQ predicts EI and changes in M_b. Importantly, the 24-h RQ value was a linear function of the fuel mix (either low-fat chow or HFD) and 24-h EI (Fig. 3*A*) and explained most of the variance in EI in each group (Table 4). Furthermore, the 24-h RQ and 24-h EI each predicted ΔM_b (Table 4); conversely, ΔM_b was a significant covariate of either 24-h RQ or 24-h EI (Fig. 3, *B* and *C*). Residual correlations of 24-h RQ and 24-h EI, after each was adjusted for ΔM_b, confirmed that their relationship was independent of shifts in energy balance, particularly in the LTDIO group (Table 4 and Fig. 3*D*).

The linear relationship between 24-h EI and 24-h RQ for each group regressed to a *y*-intercept of 24-h RQ of ~0.7, the predicted value during a fast (Fig. 3*A*). The slopes derived from the regressions were 1.24×10^{-2} (lean), 4.66×10^{-3} (STDIO), and 4.23×10^{-3} RQ units/kcal food (LTDIO). Importantly, these slopes were defined by the fuel mix and, in the case of the two HFD groups, were unaffected by feeding period ($P = 0.681$) and therefore independent of the time spent

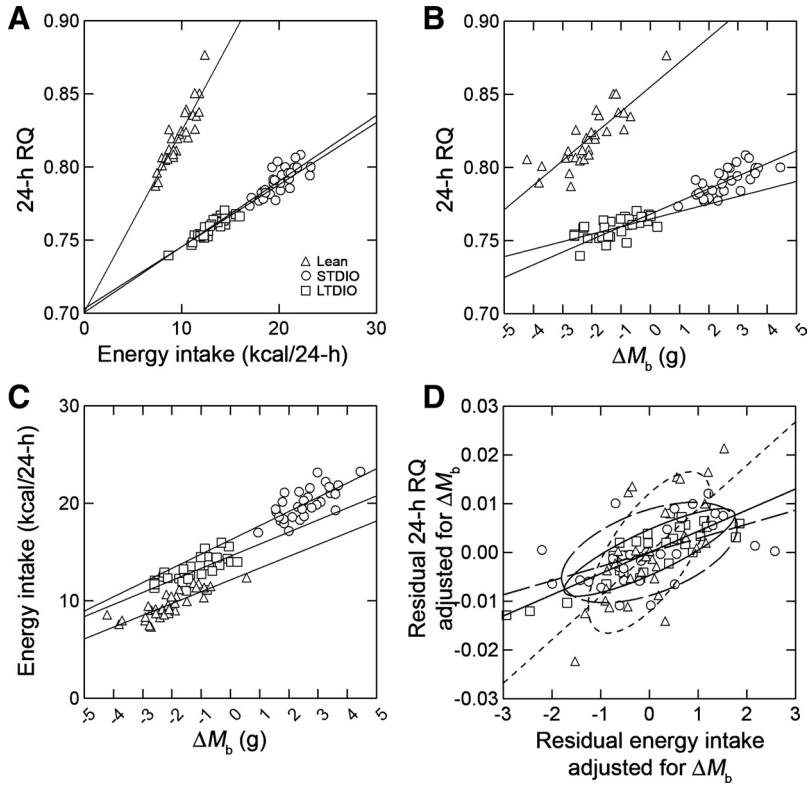

Fig. 3. *A*: scatter plot of 24-h energy intake (EI) and 24-h RQ. *B*: scatter plot of ΔM_b and 24-h RQ. *C*: scatter plot of ΔM_b and 24-h EI. *D*: double residual plot of 24-h EI and 24-h RQ (each adjusted for ΔM_b). A regression line and 70% confidence ellipse have been included for each group: short-dashed lines, lean group ($n = 32$); solid lines, LTDIO group ($n = 32$), long-dashed lines, STDIO group ($n = 32$).

Table 4. *Relationships between 24-h RQ and 24-h EI, including the influence of ΔM_b during calorimetry*

Variable		Treatment Groups								
		Lean			STDIO			LTDIO		
X	*y*	*r*	R^2	*P* value	*r*	R^2	*P* value	*r*	R^2	*P* value
RQ	EI	0.906	0.821	$<10^{-11}$	0.771	0.595	$<10^{-6}$	0.912	0.832	$<10^{-11}$
RQ	ΔM_b	0.848	0.72	$<10^{-9}$	0.739	0.546	$<10^{-5}$	0.631	0.398	$<10^{-3}$
EI	ΔM_b	0.835	0.697	$<10^{-11}$	0.725	0.526	$<10^{-5}$	0.708	0.501	$<10^{-4}$
RQ*	EI*	0.679	0.461	$<10^{-4}$	0.507	0.257	0.004	0.849	0.721	$<10^{-8}$

Relationships were determined between 24-h RQ ($\dot{V}_{CO_2}/\dot{V}_{O_2}$) and 24-h EI (kcal), including the influence of ΔM_b during calorimetry, in lean, STDIO, and LTDIO mice. Pearson's correlation coefficient, goodness of fit, and significance are given for each regression. *Adjusted for ΔM_b.

on the diet. These slopes defined a relative food quotient (RFQ) that represented the average change in RQ for each kilocalorie of food consumed during a 24-h cycle.

Substrate flexibility during early and late-stage DIO. The RQ data are alternatively displayed in Fig. 4*A*, with every RQ measurement from each group, mouse, and interval over 96-h binned by value and then converted to a relative cumulative frequency (RCF). This type of RQ transformation, first described by Riachi et al. (29), removes the factor of time and clarifies the normal distribution of RQ values. In this representation, the 50th percentile value equaled the 24-h RQ value, and the direction of energy imbalance was related to the horizontal displacement of the 24-h RQ value from the dietary FQ value; the 24-h RQ values in the lean and LTDIO groups were each shifted left (i.e., negative energy imbalance), whereas the 24-h RQ value in the STDIO group was shifted right (i.e., positive energy imbalance) (Fig. 4*A*).

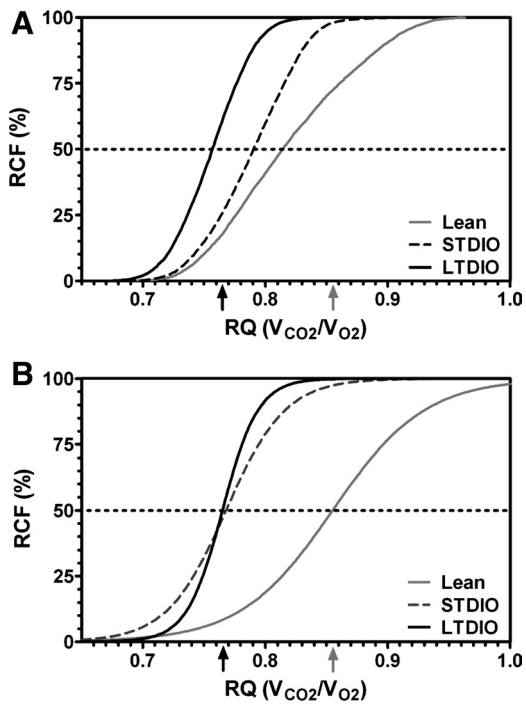

Fig. 4. *A*: RQ over 4 days transformed into relative cumulative frequency (RCF) distributions in lean, LTDIO, and STDIO mice. *B*: RCF distributions of RQ data from each group adjusted for ΔM_b. In each graph, the dashed horizontal line marks the location of the 50th percentile RQ; the location of the FQ is marked for the chow (shaded arrow, FQ = 0.855) and HFD (solid arrow, FQ =0.766).

In theory, quantitative and temporal matching between EI and EE would promote stable adipose tissue mass, resulting in a vertical 24-h RCF curve located at the FQ value of the animal's diet. By extension, the inverse of the slope of this curve, like the peak-to-peak change in RQ plotted over time from which it is derived, is a measure of both substrate flexibility and the extent of energy storage and utilization. When adjusted for ΔM_b, the curves for the STDIO and LTDIO groups intersected at their 24-h RQ values, which equaled the FQ value of the HFD (Fig. 4*B*). The greater slope in the LTDIO group demonstrated the relative loss of both substrate flexibility and degrees of lipid storage and use in the advanced stage of DIO.

The linear range of RQ values (ΔRQ) for each mouse was quantified using two methods, as the inverse Hill slopes of the RCF curves (29) and as the difference between the 95th and 5th percentile RQ values. The measurements from both methods were highly correlated (Supplemental Fig. S1), and the ΔRQ obtained from the slope method was further analyzed. (Supplemental data for this article is available online at the *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* website.)

The major determinant of the shift in ΔRQ between the lean and DIO groups was diet macronutrients [$F_{(1,94)} = 275$, $P < 10^{-10}$]. Adjusted for ΔM_b and relative to the lean group, the ΔRQ for STDIO mice had a significant 35% decrease, as the mice rapidly adapted to the HFD; the LTDIO group had a further 24% decline in ΔRQ (Table 2). However, the STDIO group still had a significantly higher ΔRQ than the LTDIO group (Table 2).

RQ range is related to EI in late-stage DIO mice. The reduction in ΔRQ following the switch from chow to HFD was overwhelmingly due to the shift in macronutrients. However, we questioned whether other variables might influence the variance in ΔRQ, since the STDIO and LTDIO groups had equivalent 24-h RQ values (adjusted for ΔM_b) but significantly different ΔRQ values.

Energy imbalances affected the ΔRQ differently in each group; ΔM_b and ΔRQ were significantly correlated in the lean ($r = 0.359$, $R^2 = 0.129$, $P = 0.047$) and STDIO groups ($r = -0.574$, $R^2 = 0.330$, $P = 0.001$) but not in the LTDIO group ($r = -0.272$, $R^2 = 0.074$, $P = 0.146$) (Supplemental Fig. S2*B*). When adjusted for ΔM_b, the only significant correlations with ΔRQ were found in the LTDIO group. First, 24-h EI was negatively correlated with, and predicted 61% of the variance in, ΔRQ (Table 5 and Fig. S2*D*). [The 24-h RQ value also predicted ΔRQ, which was unsurprising since 24-h EI and 24-h

Table 5. *Relationships between the residuals of ΔRQ adjusted for ΔM_b (y) and other variables adjusted for ΔM_b (x)*

	Treatment Groups								
	Lean			STDIO			LTDIO		
Variable *x*	*r*	R^2	*P* value	*r*	R^2	*P* value	*r*	R^2	*P* value
EE	−0.226	0.051	0.222	−0.009	0.001	0.962	−0.405	0.164	0.027
EI	0.003	0.001	0.988	0.231	0.045	0.25	−0.78	0.608	<10–6
RQ	−0.099	0.01	0.595	−0.007	0.001	0.97	−0.712	0.507	<10–4
LMA	0.037	0.001	0.844	0.299	0.089	0.12	0.279	0.078	0.136
Glucose	0.071	0.005	0.703	−0.066	0.004	0.725	0.344	0.118	0.063
Insulin	−0.308	0.095	0.092	−0.15	0.022	0.421	0.122	0.013	0.556
HOMA-IR	−0.281	0.079	0.126	−0.154	0.024	0.407	0.185	0.034	0.326

Relationships were determined between the residuals of ΔRQ adjusted for ΔM_b (*y*) and other variables adjusted for ΔM_b (*x*) in lean, LTDIO, and STDIO mice; $n = 32$ mice/group. Pearson's correlation coefficient, goodness of fit, and significance are given for each regression. Glucose level was determined in fasting blood; insulin level was determined in fasting plasma.

RQ value were strongly colinear and essentially conveyed the same information (Table 5).] Therefore, whereas the fuel composition of the HFD defined its oxidative possibilities and resulted in the 24-h RQ ≈ FQ during energy balance in each group, the ΔRQ was modulated by EI, but only in the LTDIO mice. Second, 24-h EE was negatively correlated with, and explained 16% of the variance in, ΔRQ (Table 5 and Fig. S2*C*). Therefore, in this group, greater EI was matched by an increase in EE, which narrowed the ΔRQ around the 24-h RQ value (≈FQ), indicating a greater equilibrium with ingested energy.

Surprisingly, no significant residual correlations were found between ΔRQ and other variables residuals in the lean or STDIO groups (Table 5). A negative trend was observed in the lean group between ΔRQ and fasting insulin, which explained ~10% of the variance in ΔRQ (Table 5), indicating that insulin resistance may have played a small role in restricting ΔRQ in the chow-fed mice. However, the largest effects on ΔRQ in these two groups were *1*) diet macronutrients and *2*) body mass changes during calorimetry, which dissociated EI from the ΔRQ.

DISCUSSION

This study uniquely accounted for energy imbalances during calorimetry (ΔM_b) by using least-squares linear regression; ΔM_b significantly explained variance in RQ, ΔRQ, and EI values, underscoring the major impact that shifts in energy balance can have on small animal calorimetry data. Energy imbalances varied between groups depending on the type of diet, the time on the HFD, and individual variation in EI or EE. The mice given the HFD for 4 days were hyperphagic and their weight gain was a predictable outcome (23), whereas the failure of the other two groups to maintain M_b, perhaps due to cage stress and underfeeding, clearly influenced their metabolism. Adjusting for ΔM_b controlled for variances due to energy imbalance and strengthened the statistical power and resulting interpretations.

During the transition from a low-fat diet to a HFD, the RQ rapidly decreases as the proportion of energy derived from fat oxidation increases; the 24-h RQ value will eventually equal the FQ value of the HFD once energy balance has been achieved (4, 13). The relationship between the 24-h RQ and FQ values is dependent on energy homeostasis (37). In animals balancing their intake and expenditure, fat mass is constant and the 24-h RQ and FQ values are equal. Overfeeding promotes weight gain through fat storage, shifting the 24-h RQ value above the FQ value (positive energy balance). Conversely, underfeeding results in weight loss through lipid oxidation, shifting 24-h RQ value below the FQ value (negative energy balance) (39). In the predicted energy-balanced state, the early-stage DIO mice were still hyperphagic relative to the late-stage DIO mice (Table 2) and appeared to offset their excess EI and maintain constant M_b through greater substrate flexibility; excess energy was efficiently stored during the dark period (RQ > FQ), and stored fat was readily oxidized during the light period (RQ < FQ). In contrast, the late-stage DIO mice were characterized by a loss of substrate flexibility and predictably defended their adipose mass, potentially explaining why weight loss is more difficult in DIO mice that have been switched to a low-fat diet (16) or in obese humans subjected to a restrictive dietary regimen (17, 30).

The strong relationship between the 24-h RQ and EI values highlighted the important utility of RQ as a linear indicator of not only fuel utilization but also fuel intake (Fig. 3*A*). The slope of this relationship constituted a RFQ, which was dependent on the fuel mix but independent of the duration of HFD feeding or energy status (Fig. 3*A*). The strength of this relationship lent tremendous predictive power to the 24-h RQ value. For example, in either group fed the HFD, a seemingly small shift in the 24-h RQ value of only ±0.0042–0.0047 units accounted for ±1 kcal of EI in a 24-h period.

In practice, a proper determination of RFQ requires that several assumptions and experimental conditions are met. First, the slope must regress to a logical 24-h RQ value of ~0.7 for any diet. Second, the absorption efficiency of the gut should be ~100%; ingested energy must be conserved and accounted for. [Violations of this assumption might include pathological, pharmacological, or dietary conditions that promote the passage of dietary fat in the stool (14, 27–28).] Finally, calorimetry and food intake should be monitored over strict 24-h intervals. The RFQ may be a more practical metric for assessing diets than the FQ, which has been calculated using a generalized equation from vendor-reported macronutrient ratios, particularly for diets with poorly defined composition. From a technical perspective, an empirical RFQ analysis could potentially reveal the relative obesigenic potential of any undefined diet and could be a useful benchmark for interstudy comparisons. An unresolved aspect of this analysis, requiring

further study, is the potential for the RFQ of any diet to be sensitive to differences in mouse strain or genotype.

In contrast, EI had a more complex relationship with the range of RQ values (ΔRQ), which was dependent on both diet macronutrients and, for the HFD, the duration of consumption (Table 5). Furthermore, the ΔRQ was heavily influenced by energy imbalances in the lean and short-term DIO groups, or those animals with relatively small amounts of adipose tissue. This effect was not observed in the late-stage DIO group, indicating that their additional fat mass reduced the impact of the negative shift in energy balance on substrate flexibility. Consequently, their ΔRQ was largely and negatively modulated by caloric intake and EE (Table 5), signifying a greater quantitative and temporal equilibrium between consumption and expenditure, less reliance on major shifts in energy storage and utilization, and the maintenance of fat mass at a more constant level than the lean or early-stage DIO mice.

These findings support a predictive model of energy balance in advanced DIO mice in which greater energy intake is offset by higher expenditure, reducing the amplitudes of storage and use (i.e., ΔRQ) to zero and indicating a perfect metabolism of ingested energy. Although this extreme and unlikely scenario would undoubtedly test the metabolic limits of the mice and their ability to coordinate EE with EI perfectly, it is a useful hypothetical case for understanding this model of substrate flexibility. Furthermore, the negative relationship between either EI or EE and substrate flexibility was only a long-term adaptation to the HFD; the chow-fed and short-term DIO mice had periodic cycles of greater storage and utilization, which uncoupled this relationship.

The contribution of LMA to EE in mice and whether changes in LMA influence the susceptibility to DIO have been addressed in two previous studies, with disparate results (4, 6). In one study, LMA was not different between lean and DIO mice and therefore did not explain the diet-induced weight gain (6). In another study, LMA was rapidly and sustainably reduced in mice shifted from chow to a HFD and significantly contributed to EE, suggesting that lower LMA (and by correlation, lower EE) promoted diet-induced weight gain (4). In contrast, the present study indicated that whereas LMA was lower in long-term DIO mice relative to chow-fed mice, it contributed very little to EE in either group (Table 2). (Total EE was in fact greater in the long-term DIO mice.) Therefore, the diet-induced loss of LMA did not contribute to weight gain in this study. Rather, basal metabolism and the thermic effect of food likely comprised the major components of expenditure in the lean and LTDIO groups. Greater EI was likely responsible for the long-term diet-induced weight gain (18, 24, 36). Acquired leptin resistance may have partially contributed to the lower LMA that we observed in late-stage DIO mice; leptin stimulates LMA via its actions on the arcuate nucleus (9), and LMA can be restored in hypoactive leptin-deficient mice following leptin administration (26). In addition, the effect of weight gain on the hindrance of movement cannot be ruled out.

Another important contrast with previous work (4) was that short-term HFD feeding had no impact on the level of LMA, but LMA had a significantly greater energy cost in this group. Recent work by de Wilde et al. (12) demonstrated that only 3 days on a HFD promotes greater expression in skeletal muscle of proteins defining more oxidative slow-twitch type I fibers, as well as those for oxidative phosphorylation; these characteristics were diminished after 28 days on the HFD. Therefore, the link between LMA and EE may constitute a transient adaptive mechanism that promotes energy balance, offsetting sudden and short-term increases in caloric intake and/or caloric density, similar to diet-induced thermogenesis (32).

Differences in experimental and statistical methodologies might explain the discrepancies between ours and the previous studies (4, 6). Different source vendors were used for the C57BL/6 mice, and alterations in their behavior or physiology may have developed. Each study used different low-fat chow diets of varied composition and caloric density. In addition, this study used a HFD with ~60% energy from fat, compared with ~44% (6) or ~40% energy from fat (4); variations in energy density could have stimulated different responses, and the 60% HFD may have been too energy dense to properly model DIO (7). Also, the current study adjusted for ΔM_b to address group differences in energy imbalance, some of which may have been due to stress induced by the calorimetry chambers. Importantly, in one report, EE was transformed by dividing by M_b (4), whereas this study comparatively used total EE (kcal/period) and adjusted for M_b using regression; divisional transformation of EE could lead to confounding, since it assumes that all of the variance in EE is explained by M_b (2). As a general consideration, the role of LMA in energy balance may be improperly modeled in caged systems. All of the studies examined spontaneous LMA in the cage setting, which may be less than LMA observed in the wild or when a wheel running apparatus is included, possibly altering the energetic cost of LMA (15).

Perspectives and Significance

Indirect calorimetry is routinely performed on mice to assess energy balance, but the effect of the change in body mass during calorimetry is rarely considered. This work demonstrates that the careful accounting of body mass changes during calorimetry can improve the interpretive power of the results through a simple statistical adjustment. Furthermore, it provides a framework for assigning diets a relative food quotient, which has utility in both defining the obesigenic potential of diets and improving interstudy comparisons. The reliability of the daily RQ as a strong predictor of food intake gives an added dimension to this measurement beyond its use as an indicator of fuel utilization. Finally, this work presents a new paradigm for how spontaneous activity in mice may compensate for brief increases in the intake of energy-dense diets.

DISCLOSURES

No conflicts of interest are declared by the author(s).

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