Comparison of the Effects of High-Fat Diet on Energy Flux in Mice Using Two Multiplexed Metabolic Phenotyping Systems

Jamie E. Soto1,2, Colin M. L. Burnett1,3, Patrick Ten Eyck4, E. Dale Abel1,2,5,6, and Justin L. Grobe2,3,5,6,7,8,9

Objective: Multiplexed metabolic phenotyping systems are available from multiple commercial vendors, and each system includes unique design features. Although expert opinion supports strengths and weaknesses of each design, empirical data from carefully controlled studies to test the biological impact of design differences are lacking.

Methods: Wild-type C57BL/6J mice of both sexes underwent phenotyping in OxyMax (Columbus Instruments International) and Promethion (Sable Systems International) systems located within the same room of a newly constructed animal research facility in a crossover design study. Phenotypes were examined under chow (2920x)-fed conditions and again after 4 weeks of 60% high-fat diet (D12492) feeding.

Results: Food intake, physical activity, and respiratory gas exchange data significantly diverged between systems, depending upon sex of animals and diet supplied. Estimates of energy expenditure based on gas exchange in both systems accounted for a fraction of consumed calories that was greater in males than females.

Conclusions: Design differences quantitatively impact the assessment of metabolic end points and thus the qualitative interpretation of various interventions. Importantly, current multiplexed systems remain blind to multiple additional end points, including digestive efficiency and selected forms of energy flux (nitrogenous, anaerobic, etc.), that account for a physiologically and/or pathophysiologically significant fraction of total energy flux.

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Introduction

Increased interest in comprehensive assessments of energy homeostasis for obesity research has driven field-wide adoption of multiplexed phenotyping systems, available from multiple vendors. Critically, few institutions host multiple systems from different vendors, and therefore few studies have been enabled to carefully and empirically test commonly held (but rarely tested) assumptions about the impact of different system designs. It remains unclear how studies of the same biological manipulations, when performed in distinct multiplexed phenotyping systems, may lead to differential conclusions because of the nuanced differences in phenotyping equipment.

Therefore, the current project was carried out to address the following fundamental questions: (1) Do multiplexed phenotyping systems from different vendors yield similar results and precipitate similar conclusions about the biological mechanisms manipulated, despite substantially different equipment design? (2) Does interpretation of results from studies using different systems require equipment-specific interpretation? (3) If differences in results are observed from multiple systems, how can we know whether either of the systems is correct? (4) Finally, what pitfalls and limitations should be considered when using any multiplexed system? Importantly, this study was not designed to identify mechanisms that underlie any observed differences between systems.

See Commentary, pg. 689.

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To address these questions, we examined metabolic phenotypes of wildtype C57BL/6J mice under chow-fed and 60% high-fat diet (HFD)-fed conditions using both OxyMax (Columbus Instruments International, Columbus, Ohio) and Promethion (Sable Systems International, Las Vegas, Nevada) multiplexed phenotyping systems in a longitudinal crossover design study (Figure 1).

**Methods**

**Animals and design**
Nine-week-old C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine) arrived at the University of Iowa and were acclimated for 1 week. Mice were maintained at 25°C on a 12-hour light cycle and were fed 2920 × chow diet (3.1 kcal/g, 16% of kilocalories from fat) unless otherwise noted. At 10 weeks of age, mice were randomly assigned to multiplexed phenotyping systems for 4 days (Figure 1), then returned to standard housing. At 11 weeks of age, mice were moved to the opposite system for 4 days in a crossover design. At 12 weeks of age, mice underwent body composition analyses. Animals were singly housed throughout the study to minimize effects of repeatedly regrouping males. At 16 weeks of age, mice underwent body composition analyses before and after multiplexed phenotyping. Both systems were calibrated weekly using standardized gas mixtures immediately before animals entered chambers. Ambient temperatures inside chambers were indistinguishable between systems (range observed: 29.7°C-30.8°C).

**Animal body composition**
Body composition was assessed using time-domain NMR, as previously described (1).

**OxyMax**
A 16-channel OxyMax system was used. Individual cages (interior dimensions 20.2 cm × 10.5 cm [floor] × 12.1 cm tall, with a polycarbonate mesh floor 2.7 cm above the bottom of the chamber) included a floor-mounted food hopper (10-mg resolution) and a ceiling-mounted water bottle. X- and Y-axis (horizontal plane) photoelectric beam motion detectors were positioned around the cage. Airflow was positive (500 mL/min), and gas analyses were recorded once every ~17 minutes per cage. Eight cages each were mounted inside of two thermally controlled cabinets, maintained to 30°C (to account for the lack of accessibility to bedding and thus inability to thermoregulate via burrowing behaviors).

**Promethion**
A 16-channel Promethion system was used. Individual cages (interior dimensions of 31.5 × 15.5 [floor], up to 34.5 × 19.0 [ceiling] × 13.0 cm tall) included a ceiling-mounted food hopper (3-mg resolution) and water spigot. Cages included a ceiling-mounted small “hut” into which mice could climb, which permits body mass measurements. X- and Y-axis (horizontal plane) photoelectric beam motion detectors were positioned around the cage. A running wheel accessory was available but was excluded from the current study. Airflow through the chamber was negative (2,000 mL/min), and gas analyses were recorded once per minute. Cages were mounted inside of thermally controlled cabinets that were maintained at 30°C to match parameters utilized in the OxyMax system.

**Statistics**
Although data were recorded for 4 days (Monday to Friday), only data from 6 am Wednesday to 6 am Friday were used for statistical analyses to ensure sufficient acclimation (Supporting Information Figures S1-S4 for complete, annotated gas analysis, X + Y physical activity, and cumulative food intake tracings). Metabolic rate was estimated from oxygen consumption (VO₂) and carbon dioxide production (VCO₂) rates by both systems first using the modified Weir

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**Table 1**

| C57BL/6J Mice | 2920 x Chow | 60% HFD |
|--------------|-------------|----------|
| Age (wk)     | 10          | 11       | 16       |
| 8 male       | OxyMax → Promethion | → OxyMax |
| 8 male       | Promethion → OxyMax | → Promethion |
| 7 female     | OxyMax → Promethion | → OxyMax |
| 8 female     | Promethion → OxyMax | → Promethion |

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**Figure 1** Illustration of experimental design. Male and female C57BL/6J mice fed 2920 × chow diet were subjected to a crossover design experiment evaluating metabolic phenotypes exhibited in OxyMax (Columbus Instruments International) and Promethion (Sable Systems International) multiplexed systems. Mice were examined either in OxyMax or Promethion systems at 10 weeks of age, then examined in the opposite system at 11 weeks of age. Mice were then switched to a high-fat diet, providing 60% of calories from fat (60% HFD). At 16 weeks of age, mice were again examined in the same system as employed at 10 weeks of age. Body composition was assessed using NMR at 12 weeks of age before initiating 60% HFD, and immediately before and after multiplexed system phenotyping in week 16. [Colour figure can be viewed at wileyonlinelibrary.com]
formula (2), in which heat production = 3.941(VO2) + 1.106(VCO2), or the formulas derived from Zuntz and Schumburg (3) refined by Lusk (4), in which heat production = 3.815(VO2) + 1.232(VCO2). Respiratory exchange ratio (RER) was calculated by both systems as the ratio of VCO2 to VO2. To compare generalized linear models incorporating main effects of instrument and sex with other covariates such as food intake, physical activity, total body mass, or lean body mass, the Akaike information criterion corrected for small sample sizes (AICc) was calculated to identify the most parsimonious model (5). All analytical comparisons were performed by generalized linear modeling (SAS 9; SAS Institute Inc., Cary, North Carolina), ANOVA, or t test (GraphPad Prism 7, San Diego, California) as indicated. Differences were considered significant at \( P < 0.05 \).

Figure 2 C57BL/6J mice fed 2920× chow at 10 to 11 weeks of age. (A) Body mass of mice entering OxyMax and Promethion systems. (B) Change in body mass over four 24-hour cycles. (C) Food ingested, corrected for digestible calories per gram. (D) Photoelectric beam breaks in X + Y planes. (E) Rate of O2 consumption. (F) Rate of CO2 production. (G) Heat production as estimated using the Weir equation. (H) Respiratory exchange ratio. For all end points, \( n = 16 \) males and 15 females. Comparisons performed by two-way repeated-measures ANOVA. Summary data are presented as mean ± SEM.
Results
Part 1: assessment of metabolic end points during chow feeding
Our first objective was to compare metabolic phenotypes of wild-type mice maintained on a standard chow diet, when assessed using two distinct multiplexed instruments. A crossover design approach was used, in which mice of each sex were sequentially tested in both instruments (Figure 1). The crossover design ensured that mice entering each system were matched for body mass, though as expected, males were significantly larger than females (Figure 2A). Body mass stability was assessed across the week of testing to evaluate whether either system differentially affected energy balance. A small but statistically significant difference in body mass gain was observed, with animals in OxyMax exhibiting greater weight gain (Figure 2B). Food intake was greater in the OxyMax system (Figure 2C). Major differences in cage design confounded the direct comparison of total physical activity in...
the X + Y planes. Therefore, a difference in photobeam interruptions between systems (Figure 2D) should not necessarily be interpreted as evidence of differences in activity. No differences in activity counts were observed between males versus females, and no interaction between machine and sex was observed.

Both VO₂ and VCO₂ rates were significantly increased in the males regardless of system (Figure 2E-2F). Heat production rates, estimated using either the modified Weir equation (Figure 2G) or the equation from Lusk (Supporting Information Figure S5A), were similar between systems, though males exhibited greater heat production than females. RER was lower in the Promethion system (Figure 2H). Within each sex, no difference was observed in lean body mass at week 11 between animals placed into each system (Supporting Information Figure S6).

Heat production may be influenced by factors such as body size and composition, food intake, and physical activity, and therefore we used generalized linear modeling to compare heat production in the two systems after adjusting for these covariates. Heat production, estimated using the Weir equation, remained unchanged between systems after adjustment for total body mass, lean body mass, food intake, combinations of total body mass plus food intake, or combinations of lean
body mass plus food intake (Supporting Information Table S1). The AICc method identified the adjustment for “sex, instrument, and lean body mass” as the best-fit model to account for observed variability in heat production by animals on chow diet. Importantly, because total and lean body masses between sexes differed considerably (Supporting Information Figure S6), interpretation of heat production data after normalization to total or lean body masses, when including both sexes in the model, was confounded (6); therefore, such corrections are included in the current manuscript primarily for completeness. Qualitatively and quantitatively similar results were observed when the Lusk equation was used to estimate heat production (Supporting Information Table S2).

Part 2: detection of metabolic consequences of 60% HFD feeding

Our second objective was to examine the effects of 60% HFD feeding on energy homeostasis, as assessed by the two multiplexed systems. Convergence of conclusions drawn from both systems provides confidence in conclusions regarding the effect of HFD on individual end points, whereas divergence in conclusions drawn between systems highlights context dependence of the effect of diet upon such end points.

Before and after 4 weeks of 60% HFD feeding, body composition was assessed by NMR (Supporting Information Figure S6). While all animals gained significant total, lean, fat, and fluid masses, a few minor but statistically significant differences were detected in the total and fat mass gains of individual mice randomly assigned to OxyMax versus Promethion. Males gained more mass than females, as expected.

Body masses of animals entering the phenotyping systems in week 16 were, on average within sexes, indistinguishable (Figure 3A). Over the week of testing in week 16, animals in Promethion exhibited no average change in body mass; however, a sex-dependent effect of housing in OxyMax was observed in which males gained weight while females lost weight (Figure 3B). Food intake was similar between systems, but females consumed less than males (Figure 3C). Photoelectric beam break activity was lower in Promethion (Figure 3D). In contrast to observations during the chow-feeding phase, VO₂ (Figure 3E), VCO₂ (Figure 3F), and heat production as estimated by the Weir equation (Figure 3G) or Lusk equation (Supporting Information Figure S5B) were all significantly increased in Promethion during HFD feeding. RER was similar between systems but lower in females (Figure 3H).

Heat production estimates by the Weir (Supporting Information Table S3) and Lusk (Supporting Information Table S4) equations were again adjusted for combinations of covariates using generalized linear modeling. With the exception of “sex, instrument, and physical activity” (such that instrument $P = 0.08$), the estimated heat production was significantly different between OxyMax and Promethion systems in all other statistical models, and the AICc method identified “sex, instrument, and total body mass” as the best-fit model to explain observed data.

The modified Weir equation is now more commonly employed than the equation derived from Lusk, so we took advantage of the data set to examine how the heat production estimates from each equation correlate and how covariates may impact this relationship. Reassuringly, we determined that the relationship between the results of the two equations for heat production is independent of the magnitude of heat production, diet, or the physical activity of the animals, and therefore the use of either equation to analyze the current data sets had no qualitative effect on system comparison (not shown).

Finally, we examined whether HFD feeding differentially impacted major phenotypes when assessed by the two systems. Comparing phenotypes in week 10 versus week 16 within animal demonstrated that HFD caused increased weight gain in males versus females, regardless of instrument (Figure 4A). Animals in both systems gained significant total (5.2 ± 0.5 g; $P < 0.05$ vs. zero) and lean (1.6 ± 0.2 g; $P < 0.05$ vs. zero) body masses, consumed more calories (0.08 ± 0.02 kcal/h; $P < 0.05$ vs. zero), consumed more O₂ (10.28 ± 1.41 mL/h; $P < 0.05$ vs. zero), produced more heat (0.04 ± 0.01 kcal/h; $P < 0.05$ vs. zero), and exhibited reduced RER (−0.109 ± 0.006; $P < 0.05$ vs. zero), whereas no changes in CO₂ production rate (1.28 ± 1.27 mL/h) or physical activity (1.277 ± 4.837 X + Y photoelectric beam breaks/day) were detected. Neither instrument nor sex influenced changes in lean body mass, caloric ingestion, physical activity, VO₂, VCO₂, or heat production as estimated by the modified Weir equation (Figure 4B-4G). While RER was significantly reduced by HFD feeding in both systems, the magnitude of reduction as assessed by OxyMax was greater (Figure 4H) because RER during chow feeding was higher in OxyMax (Figure 2H).

Part 3: estimating unmeasured end points contributing to energy homeostasis

Direct comparison of daily energy intake by mice in each system to estimated heat production provides an approach to estimating the magnitude of other physiological processes that are known to contribute to energy homeostasis but are not assessed in multiplexed phenotyping systems such as those employed in the current study. Subtraction of calories expended as heat from the calories ingested during chow (Figure 5A) or HFD feeding (Figure 5B) reveals a large magnitude of energy that is apparently retained by the animals. As NMR was performed immediately before and after animals were studied in week 16, changes in body composition over this week were then used to estimate the caloric equivalent of changes in fat (Figure 5C) and lean (Figure 5D) masses, using the rough estimates of 9 kcal/g for fat tissue and 4 kcal/g for lean tissue (Figure 5E). Subtracting this energy equivalent from the calories that were otherwise unaccounted for (Figure 5B) yielded an estimate of the magnitude of energy that is unaccounted for by either system through heat production plus tissue growth (Figure 5F). Calories unaccounted for in the OxyMax system averaged approximately 0.14 ± 0.05 kcal/h (21% ± 12% of total intake) versus 0.09 ± 0.03 kcal/h (17% ± 7% of total intake) in the Promethion system ($P > 0.05$ between systems, but $P < 0.05$ for either system vs. zero). This effect was due almost completely to losses observed specifically in female mice (vs. zero: $P > 0.05$ for males in OxyMax or Promethion, whereas $P < 0.05$ for females in either system). Ultimately, the Weir equation plus the caloric equivalent of growth accounted for 95% ± 5% of calories consumed during HFD feeding in males (94% ± 8% in OxyMax; 96% ± 7% in Promethion), but only 47% ± 8% for females (33% ± 12% in OxyMax; 57% ± 10% in Promethion). Future studies to dissect the interactions of sex, ambient temperature, diet, and instrumentation-specific environmental influences are warranted to understand this observation.

Discussion

With increasingly sophisticated genetic and pharmacological approaches available to manipulate biological systems controlling energy balance, there is an increased need for validation and
Figure 5 During 60% HFD feeding, not all ingested calories are accounted for by heat production as estimated by the Weir equation. (A) Calories apparently retained by mice maintained on 2920 × chow at 10 to 11 weeks of age, calculated as digestible calories consumed minus heat produced as estimated by the Weir equation. (B) Calories apparently retained by mice fed 60% HFD for 5 weeks at 16 weeks of age. (C) Change in fat mass during week 16. (D) Change in lean mass during week 16. (E) Caloric equivalent of changes in fat mass and lean mass during week 16, as estimated by multiplying fat mass change by 9 kcal/g and lean mass change by 4 kcal/g. (F) Digestible calories consumed that are not accounted for by O2/CO2 exchange using the Weir equation. In panel F, all animals combined regardless of instrument and sex (0.11 ± 0.03 kcal/h, n = 30) are significantly different from zero (one-sample t test, t = 4.1765, df = 29, P = 0.0002). Similar results were observed within OxyMax alone (0.14 ± 0.02 kcal/h, t = 2.8953, df = 13, P = 0.01) or Promethion alone (0.09 ± 0.03 kcal/h, t = 3.1383, df = 15, P = 0.007). This effect was driven by females, as unaccounted calories in males in both instruments were not statistically different from zero (0.03 ± 0.03 kcal/h, t = 0.9866, df = 15, P = 0.34), but unaccounted calories in females were different from zero regardless of instrument (0.20 ± 0.03 kcal/h, t = 6.5890, df = 13, P < 0.0001). For panels B-F, n = 8 males in OxyMax and 8 in Promethion plus 7 females in OxyMax and 8 in Promethion. Summary data are presented as mean ± SEM.
comparison of the phenotyping tools that are employed by biomedical researchers. The current study sought to clarify the nuanced differences in assessments of metabolic end points in mice studied in two commonly used multiplexed metabolic phenotyping systems. We took advantage of the fact that we recently installed two widely utilized, commercially available systems (OxyMax from Columbus Instruments International and Promethion from Sable Systems International) within meters of each other, in the same large room inside a newly constructed academic research core facility. This provided an outstanding opportunity to tightly control for environmental variables. When combined with a crossover design and a relatively large number of inbred C57BL/6J mice from a major and internationally respected commercial vendor, confidence that any observed effects could indeed be attributed to differences between the phenotyping systems is high.

Based on the data presented here, we conclude that the OxyMax and Promethion systems differ in their assessments of multiple aspects of energy flux in mice and that the differences observed are a result of the different system designs. In brief, we observed significant differences in estimates of aerobic heat production, food intake, and RER in mice studied with the Promethion system compared with the OxyMax system, depending upon diet supplied and sex of animals studied. We can only hypothesize potential causes of the observed differences, and additional nuanced studies to dissect causes for the differences observed in the current study are warranted. Differences may result directly from differential sensitivities of the systems (e.g., resolution of end point detection, end point detection limits, time resolution between measurements). Differences may also result indirectly, because of effects of system design on the cage microenvironment (e.g., the presence or absence of bedding, ambient noise from a cage fan) and study design (e.g., diet supplied, ambient temperature), which could influence subject behavior and physiology. Clearly, future studies are warranted to understand the biological mechanisms by which the array of differences in system designs (floor type, airflow rate, noise levels and types, food hopper designs, environmental enrichment, etc.) influence outcomes. Such information is required to direct future system design, though thorough investigations of such details are beyond the purpose of the current study.

It is worth noting that, despite the obvious utility of multiplexed phenotyping systems, these systems do not quantify all aspects of energy homeostasis in animals (despite marketing claims to “comprehensively” assess metabolic phenotypes). In the current study, it was estimated that while fed an HFD, a large fraction of calories consumed by the animals in either system was unaccounted for by aerobic heat production and that this difference was most pronounced in females. Multiple possible contributors to this large discrepancy exist. First, spillage of food may contribute to this discrepancy, though over a 96-hour testing period, the average 0.11-kcal/h loss of this 5.24-kcal/g HFD would equate to approximately 2.0 g of the food (D12491, which is dyed a bright blue color), which should be easily visualized in either system by technicians. Because very little spillage was noted and thus was unaccounted for in either system in the current study, spillage is unlikely to account for the bulk of these “missing” calories. Additional mechanisms that may account for these calories (and are not assessed by any current multiplexed system) may include altered digestive efficiency, protein or nitrogen metabolism, and anaerobic metabolism.

For example, no current system is designed to collect urine and feces efficiently, which is required to assess digestive efficiency. Digestive efficiency reflects the fraction of calories consumed that are absorbed by an animal (and their associated microbiota) versus lost to stool. Digestive efficiency is a major contributor to energy homeostasis, and it represents the target of the single longest-running U.S. Food and Drug Administration-approved antiobesity therapeutic compound, orlistat (Alli/Xenical) (7). Under chow-fed conditions, mice typically absorb approximately 80% of consumed calories, and under HFD-fed conditions, this fraction jumps to 90% to 95%. Dietary, genetic, surgical, and pharmacological interventions that modify energy homeostasis via changes in digestive efficiency will be completely missed when researchers employ only multiplexed phenotyping equipment such as that described herein (8). Complementary, highly quantitative methods adopted from the fields of analytical and physical chemistry, such as bomb calorimetry, are required to evaluate such end points (1).

Current multiplexed phenotyping systems are also limited by design to the detection of “aerobic” forms of energy expenditure because they all utilize the same respirometric method of measuring metabolic rate. By definition, these methods have relied upon a series of assumptions, many of which are untested or untestable (9). One such assumption is that nitrogenous and anaerobic metabolic processes are constant and indistinguishable from zero. Studies published by our group and others over the past 40 years examining these assumptions in endotherms, including mice, various birds and desert species, and humans (10-14), via the use of combined (direct calorimetry + respirometry) calorimetry systems have documented that the processes to which respirometry remains blind will typically account for between 5% and 40% of total energy expenditure. Furthermore, this contribution is highly modifiable by body composition, diet, pharmacological agents, and genetics. Recently, we determined that the gut microbiota are major contributors to this “nonaerobic” form of energy expenditure, providing up to 10% of total daily energy expenditure through mechanisms that respirometry cannot detect (15,16). Our previous studies examining the effect of a switch from chow (Teklad 7013) to a 45% HFD (D12451) in male C57BL/6J mice demonstrated that, while the “aerobic” fraction (by respirometry) accounted for only 93% of the “total” resting metabolic rate (by direct calorimetry) during chow feeding, this fraction jumped to 100% within 1 week when mice were switched to HFD (10). These findings are consistent with the current observation that, after 4 weeks of 60% HFD, aerobic heat production accounted for essentially all calories consumed in male mice, and they prompt further investigation into the contribution of aerobic versus nonaerobic processes in female mice fed various diets. Further supporting this concept and highlighting its translational significance, Pittet demonstrated in the mid-1970s that human obesity is associated with a major suppression of the nonaerobic fraction of energy expenditure, a discovery largely ignored for more than 40 years (12,13). Unfortunately, no commercial sources for direct calorimetry-based systems currently exist, and we are unaware of any reports of multiplexed direct calorimetry-based systems. This highlights a major unmet technical need for the obesity research community, as well as an opportunity for entering biotechnologists.

Nitrogen-based metabolism is an important contributor to total energy flux, and measures of nitrogen intake (in food) or urine nitrogen content are appropriately used to estimate this contributor to energy flux. Indeed, the unmodified Weir equation for estimating energy flux includes a term for nitrogen metabolism (2). Unfortunately, these methods also rely on assumptions, including that all relevant nitrogen species that are consumed are completely absorbed and that urine-based recovery is complete. Losses to the environment (e.g., through sweat or saliva) are likely minor but are almost uniformly ignored. Perhaps
most importantly, assessments of nitrogen-based metabolic processes are also necessarily performed over long time periods, and therefore the assessment of minute-to-minute changes in rates of nitrogen metabolism is impossible using these techniques. Furthermore, the caloric oxygen equivalent of all substrates is not the same. For example, Mansell and Macdonald demonstrated that, when various fuel types (including those associated with gut microbiota such as acetocacetate, hydroxybutyrate, and ethanol, in addition to dietary protein) were oxidized, the energy equivalents of consumed oxygen deviated significantly from the relationship defined by dietary fats and carbohydrates (17). These issues further underscore the need for and potential utility of direct calorimetry-based systems that do not rely solely upon gas respirometry-based estimates of energy expenditure.

The discovery that both OxyMax and Promethion systems fail to account for any large fraction of total energy turnover is itself of importance to the field. In addition, the determination that there is a different detection of the magnitude of this missing fraction in males versus females is also of interest and may contribute to sex differences in weight gain (Figure 5F). It is possible, given the different design of food supply mechanisms in each system, that differences in spillage recovery may contribute to observed differences. It is possible that this difference may represent sex-specific alterations in digestive efficiency. It is also conceivable that, because animals in the Promethion system live in a standard bedding floor and therefore have access to feces whereas animals in the OxyMax system are housed on a suspended floor that restricts access to feces, sex-dependent differences in coprophagic behaviors and tendency to consume bedding materials may impact measurable food intake behaviors and digestive efficiency and thus differentially impact the fraction of calories that is accounted for by the two systems.

The primary purpose of the current study was to evaluate the impact of employing two different commercially available multiplexed phenotyping systems on assessments of energy homeostasis. As a result, the experimental paradigm was optimized to detect differences between systems rather than to optimize the external validity of either system’s approach. Therefore, there are an array of important study limitations that must be noted. For example, because mice thermoregulate in part through burrowing behaviors, and because the Promethion system provides access to bedding material but the OxyMax system does not, it was decided that energy homeostasis assessments would be performed in both systems while maintained with a thermoneutral 30°C chamber ambient temperature. This elevated ambient temperature choice may conceivably minimize differences that could be detected between systems, and future comparisons of the two systems at standard housing temperatures (22°C-25°C) may yield additional useful information with regard to the use of these multiplexed systems. Furthermore, the use of a 30°C ambient temperature may conceivably contribute to the observed “unaccounted for” calories in females fed an HFD (Figure 5F) through effects on body composition (Figure 5B-5E). Future studies need to be performed to carefully quantify such missing calories in both sexes at various temperatures and in the context of various diets.

Additionally, it should be noted that no separate acclimation period was provided for animals entering either system, though both manufacturers suggest that users include acclimation phases. Our study design was purposeful, as we intended to empirically document the time course of acclimation in either system, both to test the effects of acclimation and to compare acclimation time courses between systems. Indeed, from the current study, it appears that 48 hours of acclimation is sufficient to establish normal rhythmicity in most or all end points in either system (Supporting Information Figures S1-S4). Nonetheless, the external validity of this acclimation time course requires further study in the context of various covariates.

Many additional comparisons between systems are possible to further dissect the influence of system design on end point evaluation. For example, it is apparent from close examination of full time-course tracings (Supporting Information Figures S1-S2) that larger differences in gas exchange between light and dark phases were observed from subjects examined in the Promethion system, despite no differences in 24-hour averages (Figures 2,3), though no formal statistical comparison of these possible amplitude changes was performed herein. Ambient temperature and psychological stress have both been associated with alterations in such rhythmicity (18,19), which underscores yet another area that requires future establishment of the external validity of either multiplexed system.

Finally, for the purpose of the current study, the modified Weir and Lusk equations were utilized to estimate aerobic heat production. Hall et al. recently described an updated equation that may provide even more accurate estimates, in which the proportion of total energy expenditure accounted for by protein metabolism can be estimated from the proportion of protein in the diet (20). It is logical that the use of this newer equation will likely modify the quantitative results comparing each phase of the current study (i.e., 2920 × chow diet provides 24% of kilocalories from protein; D12492 provides 20% of kilocalories from protein); however, the use of any other equation would not be expected to have substantial effects on the comparison of OxyMax versus Promethion systems within either phase. Again, continued refinement of technologies and equations used to estimate heat production from simple to measure end points such as oxygen and carbon dioxide exchange from animals of each sex, in the context of varied diets, ambient temperatures, etc., as well as in various species, is warranted.

Conclusion

In conclusion, our studies support the ongoing and major utility of both OxyMax and Promethion phenotyping systems, but they also highlight critical quantitative differences that result from the use of each system, as well as complex diet- and sex-dependent effects on measures of energy flux in each system. Caution is therefore advised for researchers directly comparing results (quantitative and qualitative) of studies examining animals in each distinct type of multiplexed system. The use of either single system in isolation may lead to results that contradict results obtained from a different system type, primarily because of system design rather than because of biological mechanisms. Such differences may help explain contradictory results reported by users at independent research institutions. It is important to appreciate that work to establish the external validity of either system is ongoing by various groups and is required to clarify the impacts of sex, diet, ambient temperature, noise, and many other variables on energy flux. The current study provides preliminary data that may prompt hypotheses to explain the observed differences in results obtained between systems; however, it is critical to recognize that the current study was not designed to test causal relationships and therefore cannot be used to directly justify the superiority or validity of either design. Finally, researchers must be
cognizant of the inability of any current multiplexed system to detect or measure multiple additional physiologically and pathophysiologically significant mechanisms that contribute to energy homeostasis, such as digestive efficiency and nonaerobic metabolic processes. Additional work is needed to understand the validity, strengths, weaknesses, and interactive effects of multiplexed system designs in studies of metabolic physiology.

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