

Reduced Non-Exercise Activity Attenuates Negative Energy Balance in Mice Engaged in Voluntary Exercise

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ABBREVIATIONS

EE – energy expenditure

VWR – voluntary wheel running

OWA – off wheel activity

EE_{rest} – EE during inactivity

EE_{VWR} – EE during voluntary wheel running

EE_{OWA} – EE during off wheel activity

EE_{total} – EE_{rest} + EE_{OWA} + EE_{VWR}

ME – Metabolic efficiency

J_{O_2} – O₂ consumption by permeabilized muscle fibers

J_{ATP} – ATP production by permeabilized muscle fibers

ATP/O – oxidative phosphorylation efficiency of permeabilized muscle fibers

ABSTRACT

Exercise alone is often ineffective for treating obesity despite the associated increase in metabolic requirements. Decreased non-exercise physical activity has been implicated in this resistance to weight loss, but the mechanisms responsible are unclear. We quantified the metabolic cost of non-exercise, or “off-wheel”, activity (OWA) and voluntary wheel running (VWR) and examined whether changes in OWA during VWR altered energy balance in chow-fed C57BL/6J mice (n=12). Energy expenditure (EE), energy intake, and behavior (VWR and OWA) were continuously monitored for four days with locked running wheels followed by nine days with unlocked running wheels. Unlocking the running wheels increased EE as a function of VWR distance. Metabolic cost of exercise (MCE; kcal per meter traveled) decreased with increasing VWR speed. Unlocking the wheel led to a negative energy balance, but also decreased OWA, which was predicted to mitigate the expected change in energy balance by ~45%. A novel behavioral circuit involved repeated bouts of VWR and roaming was discovered and revealed novel predictors of VWR behavior. The integrated analysis described here reveals that weight loss effects of voluntary exercise can be countered by a reduction in non-exercise activity.

INTRODUCTION

Physical exercise alone generally fails to result in meaningful weight loss in obese populations (1; 2). Similarly, numerous reports suggest that the same is true regarding the effectiveness of voluntary wheel running (VWR) for inducing weight loss in mice (3-5). The ineffectiveness of voluntary exercise for weight loss is, at least in part, due to compensatory feedback mechanisms that counterbalance increased energy expenditure (EE) and preserve energy balance. Chronic compensation to exercise (> 2 weeks) includes increased food intake (6; 7), reduced resting metabolic rate (8-10) and decreased non-exercise physical activity (11). Acute compensation to exercise (< 2 weeks) in mice can be independent of increased food intake (12), suggesting that a decline in non-exercise physical activity may help offset exercise-induced EE in accordance with a “constrained” model of EE (13). Indeed, mice engaged in voluntary wheel running (VWR) have decreased non-exercise physical activity, termed off-wheel activity (OWA) (14-16). The metabolic impact of decreased OWA with VWR has not been previously reported.

There is limited data regarding the contributions of VWR and OWA to EE in mice. This is because VWR and OWA are voluntary and typically performed at low to moderate intensity, thus requiring sensitive instrumentation to detect an increase in EE (17). Only one report to date has demonstrated an acute (within minutes) effect of VWR on EE and was done in outbred deer mice (18). O’Neal et al. (14) were able to detect a contribution of VWR to the daily energy budget in male C57 mice during a short duration (3 days) study and observed a diminishing contribution of VWR to EE during a longer duration (21 days) study. Surprisingly however, the increase in EE from VWR observed by O’Neal et al. (14) during their chronic study was not positively correlated with VWR distance. Brown et al. (19) observed a distance-dependent

increase in daily VO_2 in female C57BL/6N mice, but a caveat to this study is that female mice run much further than males (20). While few studies have directly measured EE during VWR, no studies to date have been able to detect the independent contribution of OWA to EE (21); thus, potential interactions between VWR and OWA and their impact on EE and energy balance have not been addressed.

There is significant variability in voluntary physical activity patterns in mice, even within an inbred strain (14). Skeletal muscle (SkM) mitochondria are the primary source of ATP during low to moderate intensity exercise, and exercise training has been shown to improve SkM mitochondrial efficiency (22). Recent work has revealed that muscle mitochondrial efficiency also acutely increases as a function of metabolic demand (23), suggesting that this variable may be a novel factor explaining differences in VWR behavior between mice.

The purpose of the studies described here was to combine indirect calorimetry, context-specific behavioral mapping and mitochondrial energetics in C57BL/6J mice to: 1) quantify the metabolic contributions of VWR and OWA to EE, 2) examine whether reduced OWA is a mechanism to preserve energy balance in response to VWR and 3) identify behavioral and metabolic factors that explain variance in VWR between mice.

METHODS

Animal Housing and Study Design

Vanderbilt University Animal Care and Use Committee approved all experiments. Twelve-week old male C57BL/6J mice (n=12) were purchased from Jackson Laboratories and housed in the

Vanderbilt University Mouse Metabolic Phenotyping Center. Mice were singly housed starting at 15 weeks of age. Indirect calorimetry studies were performed from 19-21 weeks of age. At 23 weeks of age, following a four-hour fast and under isoflurane inhalation, red gastrocnemius (RG) was excised for mitochondrial energetics assays.

Indirect Calorimetry and Voluntary Physical Activity Behavior

Mice were singly housed in a Promethion Metabolic Analyzer (Sable Systems, North Las Vegas, NV) at ambient temperature (20-22°C). Cages contained Pure-O'Cel bedding (The Andersons Inc, Maumee, OH) and mice had *ad libitum* access to 5L0D (LabDiet, St. Louis, MO) chow diet and water throughout the study. Running wheels were present for the entire time period, but were locked for the first 4 days. O₂ and CO₂ were continuously monitored for assessment of EE. Mouse cage behavior, including roaming (XYZ beam breaks), food and water intake and VWR were monitored continuously with second-to-second resolution. Contextual behavioral mapping was achieved by integrating XYZ beam break data with mass measurement modules for food intake, water intake and body weight. Additional technical information is provided in **Supplemental Materials**.

Behavior mapping analysis for each animal was performed for the last dark phase (12 continuous hours) of each condition (locked or unlocked wheel) using Expedata software (Sable Systems, North Las Vegas, NV). This analysis yielded a complete sequential list of behaviors. For each behavior, the duration and frequency was measured. From the sequential list of behaviors, time budgets were generated as percentages. Time budget data were analyzed with two-dimensional hierarchical clustering (x-axis = animal, y-axis = behavior) and visualized as heat maps

representing individual behavior budgets for individual mice relative to the group mean. Behavioral transitions were determined for the last continuous 24 hours of data for each condition and probability matrices were visualized as a Markov chain.

SkM mitochondrial O₂ consumption, ATP production and oxidative efficiency (ATP/O)

Mitochondrial ATP production, O₂ consumption and oxidative efficiency (ATP/O) were measured in permeabilized myofiber bundles (PmFBs) from red gastrocnemius muscle as previously described (23).

Calculations

The contributions of OWA or VWR to EE_{total} were determined from time-matched behavior and metabolic data collected every 5 minutes over the last 72 hours (435 data points/mouse) of each period (i.e. wheel locked or unlocked). Individual data points were binned based on OWA or VWR activity speed. EE_{rest} (i.e. activity = 0) was determined then subtracted from EE during OWA or VWR. The remaining EE was multiplied by the percentage of time spent active at a given speed to determine EE_{OWA} or EE_{VWR} . To determine EE_{OWA} in mice with an unlocked running wheel, frequency distributions of OWA speed were generated as described above. Notably however, the fraction of EE above rest in mice with a locked wheel was used instead of that measured when the wheel was unlocked. This was done because VWR typically occurred in concert with OWA, thereby convoluting the specific contribution of OWA to EE. This calculation relies on the assumption that the energetic cost of OWA at a given speed does not change when the wheel was unlocked. Metabolic cost of exercise (MCE) of VWR was calculated by dividing the total kcal expended at a given binned speed by the total distance traveled at that

speed. The average of energy cost of VWR across all speeds was used to calculate MCE in kcal/m for each individual mouse.

Energy Intake (kcal) = Food Intake (g) * 2.91 (kcal/g). 2.91 kcal/g represents the metabolizable energy obtained from the diet based on manufacturer data. Daily Energy Balance = Energy Intake (kcal/day) – Energy Expenditure (kcal/day). Δ OWA, Δ EE, Δ Energy Intake and Δ Energy Balance were each calculated as (unlocked wheel – locked wheel).

Statistics

Paired Student's t-test was used to compare data when the wheel was locked versus unlocked. One-way ANOVA with Tukey post-hoc test was used to examine rate-dependent metabolic responses. Simple or multiple linear regression analysis was used to generate correlation coefficients (r) or coefficients of determination (r^2) as appropriate. F-test was used to determine statistical significance of linear regressions. Unless otherwise indicated, all data are presented as the mean +/- SD. Statistical significance was defined as $p < 0.05$.

RESULTS

Daily light and dark phase VWR for the entire experimental period is shown in **Figure 1A** and the average from Days 11-13 (shown in **Figure 1B**) was used for subsequent analyses. There was no significant relationship between VWR distances during the light phase and the dark phase (**Figure 1C**). EE_{total} for the entire experimental period is shown in **Figure 1D**. EE_{total} increased during both light and dark phases when the running wheel was unlocked (**Figure 1E**). The change in dark phase EE_{total} (ΔEE_{total}) closely correlated with VWR distance (**Figure 1F**).

The relative amount of time mice spent at rest or performing VWR at different speeds is shown in **Figure 2A**. EE_{total} normalized to time (kcal/hr) was significantly elevated when VWR was performed at any speed relative to inactivity (**Figure 2B**). EE_{total} linearly increased as a function of VWR speed ($r^2 = 0.96$, $p < 0.0001$; Slope = 3.0 ± 0.4 kcal/km (Mean +/- SEM)). The absolute contribution of VWR to EE (EE_{VWR}) at a given speed is shown in **Figure 2C**. Summing these individual contributions, we concluded that VWR accounted for $26.6 \pm 7.7\%$ of EE_{total} . MCE (kcal/m) (**Figure 2D**) decreased as VWR speed increased and tightly fit a one-phase exponential decay curve ($r^2 > 0.98$ for all 12 mice). MCE at < 5 m/min was moderately positively correlated with VWR distance ($r^2 = 0.47$; $p < 0.05$) but MCE at higher speeds of VWR were not significantly correlated. These data reinforce the observation made in **Figure 1** that VWR is a direct contributor to EE and implicate MCE at low VWR speeds as a predictive indicator of VWR behavior.

We hypothesized that SkM OXPHOS efficiency would be positively related to physical activity, thus providing a link between efficient aerobic metabolism and the motivation to exercise. J_{ATP} , J_{O_2} and the resulting ATP/O measured as a function of [ADP] is provided in **Supplemental Materials**. Surprisingly, plotting ATP/O at each [ADP] studied (5, 20, 75 and 200 μM) against VWR distance, ΔEE_{total} or MCE did not reveal any significant relationships ($r^2 < 0.1$ for all ATP/O values tested). These data suggest that SkM mitochondrial oxidative efficiency is not associated with VWR performed over a relatively brief period of time.

Dark phase OWA was decreased in mice with an unlocked running wheel (**Figures 3A and B**), but this was not related to VWR distance (**Figure 3C**) or time spent performing VWR ($r^2 = 0.10$; $p = 0.31$). Energy intake was not different in the light or dark phases when the running wheel was unlocked (**Figures 3D and E**) or related to VWR (**Figure 3F**). Daily energy balance became negative when the running wheel was unlocked (**Figures 3G and H**), but changes in energy balance were not significantly related to dark phase VWR distance (**Figure 3I**).

To explore how unlocking the running wheel might lead to decreased OWA, we compared the decline in OWA (Δ OWA) to changes in key metabolic variables. Δ OWA was not related to Δ EE_{total} (**Figure 3J**) or Δ EI (**Figure 3K**), but was positively associated with Δ EB (**Figure 3L**). These data suggest that reduced OWA may partially offset negative energy balances in response to the increased EE of VWR.

Frequency distributions of OWA speed (**Figure 4A**), EE_{total} as a function of OWA rate (**Figure 4B**) and EE_{OWA} (**Figure 4C**) binned in 0.1 m/min increments were calculated. EE_{OWA} was not related to total OWA distance (**Figure 4D**). EE_{OWA} measured at speeds lower than 0.3 m/min also failed to correlate with OWA distance (**Figure 4E**). EE_{OWA} at speeds greater than 0.3 m/min was positively correlated with OWA distance (**Figure 4F**). We next examined food intake and water intake as they related to OWA and EE. The percentage of time spent eating was greater at low OWA (**Figure 4G**) whereas time spent drinking was greater at high OWA (**Figure 4H**). Rates of food and water intake were both greater, as expected, at low compared to high OWA (**Figures 4I and J**). Food intake corresponded to a ~30% increase in EE_{total} during low OWA but only a ~3% increase during high OWA (**Figure 4K**). Similar effects were observed for

water intake (**Figure 4L**). These data demonstrate for the first time that OWA is an independent contributor to EE_{total} in mice. Since the contribution of OWA to EE_{total} was only observed at OWA speeds above 0.3 m/min, it is reasonable to conclude, as others have (21), that OWA at low speeds is not a direct contributor to EE.

EE_{OWA} when OWA was > 0.3 m/min decreased when running wheels were unlocked (**Figure 5A**). This decrease in EE_{OWA} was linearly related to the decrease in OWA distance (**Figure 5B**) confirming that it represents a change in the direct contribution of OWA to EE_{total} . We next compared the measured EE_{total} in mice with an unlocked running wheel to the predicted EE_{total} if OWA were not reduced. The formula for this calculation was: Predicted $EE_{total} = \text{Measured } EE_{total} + (EE_{OWA} \text{ Locked} - EE_{OWA} \text{ Unlocked})$. This calculation predicts that, if OWA was not reduced, EE_{total} would be reduced $\sim 5\%$ (**Figure 5C**) and energy balance would be $\sim 45\%$ less negative (**Figure 5D**). These data are the first to demonstrate a direct impact of reduced non-exercise physical activity on exercise metabolism.

Since OWA impacts EE_{total} and energy balance, we sought to define which OWA behaviors are altered by VWR. A behavioral analysis approach that quantifies the relative amount of time a mouse spends performing various activities was used. When the wheel was unlocked, mice spent $17.71 \pm 5.4\%$ (Mean \pm SD) of their time on the wheel. Time spent running was positively correlated with VWR distance ($r^2 = 0.86$; $p < 0.0001$) and negatively correlated with time spent roaming ($r^2 = 0.92$, $p < 0.0001$). Notably however, the change in time spent roaming (unlocked – locked) was not correlated with the change in OWA distance ($r^2 = 0.11$; $p = 0.29$), suggesting more intricate remodeling of OWA in response to VWR. To first examine changes in OWA

elicited by unlocking the wheel, independent of wheel running behavior, time spent running was removed as a variable. As a group, mice spent more time performing short roaming, and less time performing long roaming (**Figure 6A**) when the wheel was unlocked. The relative change in behavioral frequency for each mouse (unlocked/locked) was visualized as a two-dimensional hierarchical cluster (**Figure 6B**), revealing a consistent increase in time spent short roaming. Mice performed long roaming at a slower speed when the wheel was unlocked with no change in short roaming speed (**Figure 6C**). The total distance traveled during long roaming was reduced ~65% when the running wheel was unlocked (**Figure 6D**). Despite a decrease in long roaming distance, VWR distance was correlated with time spent short roaming, but not long roaming (**Figure 6E**). Plotting the percent change in short roaming in response to an unlocked wheel against VWR distance revealed an even stronger relationship (**Figure 6F**). These findings demonstrate that the impact of VWR on OWA is intricate and multi-faceted.

We further investigated the relationship between short roaming and VWR using behavioral transition matrices. With this approach, we tested the hypothesis that short roaming and VWR constitute a repeated behavioral circuit that predicts VWR distance. Behavioral transitions (data provided in **Supplemental Materials**) were visualized as a Markov chain (**Figure 7**). Consistent with our hypothesis, short roaming was the most common behavior preceding VWR, occurring 39% of the time, and VWR was the most common behavior that preceded short roaming, occurring 86% of the time. Bouts of short roaming adjacent to (before or after) VWR bouts were performed at a higher speed than other short roaming bouts (0.91 ± 0.14 vs. 0.62 ± 0.15 cm/sec, $p < 0.0001$). The frequency that short roaming preceded VWR was a positive predictor of VWR distance ($r^2 = 0.54$; $p < 0.01$). These findings led us to hypothesize that the number of

repeated running bouts would predict VWR distance. Surprisingly, the total number of repeated running bouts did not predict VWR distance ($r^2 = 0.23$; $p = 0.12$). Average VWR bout duration was tightly correlated with VWR distance ($r^2 = 0.59$; $p < 0.01$). Long, repeated VWR bouts (>100 seconds) positively predicted VWR distance ($r^2 = 0.72$; $p < 0.001$) whereas no relationship was observed between VWR distance and the number of short repeated running bouts (< 100 seconds) ($r^2 < 0.1$). These data demonstrate functional interactions between VWR and OWA that more comprehensively describe voluntary exercise behavior.

DISCUSSION

In this report we characterize metabolic and behavioral remodeling that occurs in mice engaged in VWR. We have quantified the direct contributions of VWR and OWA to EE and found that decreased OWA can preserve energy balance in response to VWR. Remodeling of OWA is complex and includes both gross reductions in OWA distance and time spent roaming, but also changes in the duration and context of roaming behavior. These intricate and assorted modifications in cage behavior suggest an important interplay between VWR and OWA in the regulation of energy balance and provide a framework for assessing mouse metabolism in the context of both exercise and non-exercise behavior.

VWR contributing to EE_{total} (**Figure 1D-F**) is consistent with previous work in outbred mice (18; 24), as well as a recent study from C57BL/6 mice (14), but at odds with another report (21). We refine our understanding of the relationship between VWR and EE_{total} by showing a strong positive linear correlation between dark phase EE_{total} and VWR distance (**Figure 1F**). One reason a relationship between VWR and EE_{total} has been difficult to detect may relate to the

interval of metabolic gas sampling. For example, Chappell et al. (18) used a fast sampling interval (90 seconds) and demonstrated a speed-dependent contribution of VWR to EE_{total} . In the present study, a 5-minute sampling interval was used and we observed a similar relationship between VWR and EE_{total} . O'Neal et al. (14) used 13-minute intervals and observed an increase in daily EE_{total} when mice had an unlocked wheel, but the rise in EE_{total} was not correlated with VWR distance. Finally, Virtue et al. (21) used 18-minute intervals and failed to detect a contribution of VWR to EE_{total} in mice. Thus, sampling rate is a critical factor in the ability to resolve the contribution of VWR to EE_{total} .

We were also able to detect the acute (5 minutes) contribution of VWR to EE_{total} (**Figure 2A**). From these data, we found that MCE decreased as a function of VWR speed (**Figure 2D**) and was positively related to VWR distance. This observation suggests that metabolic efficiency may be a predictor of voluntary physical activity behavior and warrants further investigation. SkM mitochondrial oxidative efficiency was not related to VWR measured over this relatively brief time period (**Supplemental Materials**), suggesting that differences in MCE may be primarily due to biomechanic or cardiovascular differences as opposed to variations in oxidative metabolism *per se*.

As far as we are aware, this is the first study to report an independent contribution of OWA to EE_{total} . From these data, we also provide a potential explanation for why previous reports have been unable to do so. For example, Virtue et al. (21) found a linear relationship between EE_{total} and low speeds of OWA. Examining only low OWA rates to determine EE_{OWA} , they found no significant relationship between EE_{OWA} and OWA speed and thus concluded that the

independent contribution of OWA to EE_{total} was essentially zero. Our data show the same poor relationship between EE_{OWA} at low OWA speeds (< 0.3 m/min) and total OWA distance (**Figure 4E**), likely due to concurrent intake of food and/or water (**Figures 4K and L**). However, we demonstrate that EE_{OWA} at > 0.3 m/min is positively correlated with OWA distance (**Figure 4F**) where food/water intake have minimal effect on EE. These data collectively suggest that food and water intake explain, at least in part, why previous reports have been unable to detect an independent contribution of OWA to EE_{total} .

A primary goal of the experiments performed was to define the impact of decreased OWA on energy balance in mice performing VWR. In these experiments, an unlocked wheel decreased energy balance (**Figures 3G and H**) independent of VWR distance (**Figure 3I**) and changes in energy intake (**Figure 3B**). A lack of change in energy intake has been reported in mice that engage in VWR for brief periods of time (14). The negative energy balance observed with an unlocked wheel was associated with a decrease in OWA (**Figure 3L**) but not VWR distance (**Figure 3I**), EE_{total} (**Figure 3J**) or energy intake (**Figure 3K**). These findings collectively suggest that energy balance may be a determinant of OWA in mice performing VWR.

We observed a reduction in EE_{OWA} at high OWA speeds (i.e. > 0.3 m/min) when the running wheel was unlocked compared to when it was locked (**Figure 5A**). This change in EE_{OWA} was linearly related to the change in OWA distance resulting from the wheel being unlocked (**Figure 5B**), reflecting a decrease in the direct contribution of OWA to EE_{total} . The decrease in EE_{OWA} when the wheel was unlocked is estimated to reduce EE_{total} by $\sim 5\%$ and consequently mitigate the drop in daily energy balance by $\sim 45\%$ (**Figures 5C and D**). This difference in energy

balance, specifically due to reduced OWA, translates to a decrease in weight loss of 0.4 g of fat per week. These data are consistent with a “constrained” model of EE_{total} where reduced non-exercise activity serves to maintain a ceiling for EE (13; 25). Human studies examining whether non-exercise physical activity decreases with exercise are somewhat inconsistent (reviewed in (26)), so it is possible that our approach can be coupled with mouse genetic engineering to better understand these variable responses.

VWR behavior is highly variable between individual mice, even within an inbred strain (14). OWA behavior of the C57BL/6J mice studied here dramatically changed when they were allowed to run on a wheel. For example, mice spent less time roaming when given access to a wheel. Specifically, long roaming was performed less often (**Figure 6A**) and at a slower speed (**Figure 6C**) in mice with an unlocked wheel. These changes resulted in a ~65% decrease in distance traveled during long roaming (**Figure 6D**). Meijer and Robbers (27) recently showed that wild mice run significant distances on a wheel placed in their natural environment for no clear reason, so it is interesting to consider whether this observation could be explained by VWR and long roaming being functionally similar behaviors associated with foraging.

Mice performing VWR engaged in a distinct behavioral circuit consisting of repeated bouts of short roaming and VWR (**Figure 7**). Engaging in this circuit was found to be a positive predictor of VWR distance, suggesting it may be a tractable mechanism for better understanding voluntary exercise behavior. One possible explanation for the existence of this circuit may be to avoid overexertion incurred by longer bouts. These findings provide valuable insight into our

understanding of why mice, and potentially humans, have such broad variability in their willingness to engage in voluntary exercise and present new behaviors to interrogate.

The findings described here should be considered with the following caveats. The ability to assess activity-induced changes in EE was limited by the temporal resolution of gas analysis (5 minute intervals). These studies examined a relatively acute timeframe for VWR and it is unknown whether these behavioral changes persist over longer periods of time. Chow-fed male mice were studied, so it is unknown whether obese mice in positive energy balance would respond similarly. Finally, it is unknown whether female mice have the same behavioral and metabolic responses to VWR.

The use of rodent models to study voluntary metabolic and behavioral responses to VWR has been constrained by an inability to detect the contributions of VWR and OWA to EE, distinguish between context-specific behaviors, and account for individual variability in VWR performance within a group of animals. By addressing these gaps in our understanding of voluntary exercise metabolism and behavior, our findings (summarized in **Figure 8**) provide the most comprehensive assessment to date of the metabolic and behavioral response to voluntary exercise and provide a framework for deconstructing the complex regulation of energy balance in mice.

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studies. All authors contributed to manuscript writing and have approved the final version. D.S.L. is the guarantor of the work.

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FIGURE LEGENDS

Figure 1: Voluntary wheel running elicits a distance-dependent increase in energy expenditure. VWR was measured during the light (open boxes and circles) and dark (closed boxes and squares) phases over the entire experimental period (**A**) and averaged over the final three days (**B**). %CV = coefficient of variation. No relationship was observed between light phase and dark phase VWR (**C**). EE_{total} was measured during the light (open boxes) and dark phases (closed boxes) over the entire experimental period (**D**). EE_{total} increased in both the light (circles) and dark (squares) phases of mice with access to a running wheel (**E**). The change in EE_{total} (ΔEE_{total}) after unlocking the wheel was tightly correlated with VWR distance (**F**). N = 12 mice; * indicates $p < 0.05$ between bracketed groups using paired Student's t-test. Regression lines are shown for relationships with $p < 0.05$ with F-test.

Figure 2: Acute changes in energy expenditure and metabolic efficiency as a function of voluntary wheel running speed. Real-time data from each mouse (435 data points/mouse) over the final three dark phases of the experimental period were separated based on running speed over each five-minute interval. The fraction of time mice spent inactive (VWR = 0) or running at different VWR speeds was measured (**A**). EE_{total} of mice during inactivity or at various VWR speeds was determined (**B**). The fraction of EE_{total} attributed to VWR at a given speed (active EE – inactive EE) was multiplied by the time spent at that speed to determine the contribution of VWR to EE_{total} (EE_{VWR}) (**C**). Metabolic cost of exercise (MCE) (kcal/m traveled) was determined by dividing EE_{VWR} by the distance traveled at each speed (**D**). N=12 mice; * or # indicates $p < 0.05$ between bracketed groups.

Figure 3: Energy balance is a determinant of the compensatory reduction in off-wheel physical activity in mice performing voluntary exercise. OWA was measured over the entire experimental period (A). Average dark phase OWA decreased in mice with an unlocked running wheel (B) but was not correlated with VWR distance (C). Energy intake was measured over the entire experimental period (D) and was not different when the running wheel was unlocked (E) or correlated with VWR distance (F). Daily energy balance (Energy Intake – EE) was measured over the entire experimental period (G). Average daily energy balance became negative in mice performing VWR (H) but was not correlated with VWR distance (I). The change in OWA when the running wheel was unlocked (Δ OWA) was not correlated to Δ EE (J) or Δ Energy Intake (K) but was positively related to Δ Energy Balance (L). N=12 mice; * indicates $p < 0.05$ between bracketed groups using paired Student's t-test. Regression lines are shown for relationships with $p < 0.05$ with F-test.

Figure 4: Off-wheel activity (OWA) is an independent contributor to energy expenditure in mice. Time spent performing OWA at various speeds (A), EE_{total} when performing OWA (B) and EE_{OWA} (C) were determined from 435 data points collected during three consecutive dark phases of data (Days 2-4). OWA distance was plotted against total EE_{OWA} (D), EE_{OWA} with OWA < 0.3 m/min (E) and EE_{OWA} with OWA > 0.3 m/min (F). Time spent eating (G) was greater when OWA is low (< 0.3 m/min) compared to high (> 0.3 m/min) whereas the opposite was true for time spent drinking (H). Average intake rates of food (I) and water (J) were both greater during low OWA compared to high OWA. EE_{total} during food intake (K) and water intake (L) were both increased at both high and low rates of OWA but the effect on EE was much larger at low rates of OWA. * indicates $p < 0.05$ compared to OWA = 0, # indicates $p <$

0.05 compared to OWA < 0.1 m/min, † indicates $p < 0.05$ compared to OWA < 0.3 m/min using ordinary one-way ANOVA with Tukey post-hoc test; All other panels) * indicates $p < 0.05$ between bracketed group using paired Student's t-test.

Figure 5: Reduced off-wheel activity mitigates negative energy balance in mice performing voluntary wheel running exercise. EE_{OWA} when OWA > 0.3 m/min decreased in mice with an unlocked running wheel (A). ΔEE_{OWA} when OWA > 0.3 m/min was positively related to the change in OWA distance when the wheel was unlocked (B). If OWA was not decreased, it is predicted that EE_{total} would be ~5% greater (C) and energy balance would be ~45% more negative (D) than measured. N=12 mice. * indicates $p < 0.05$ between groups using paired Student's t-test.

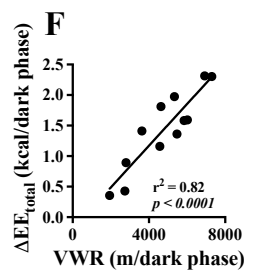
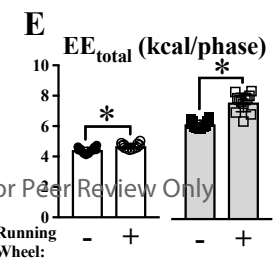
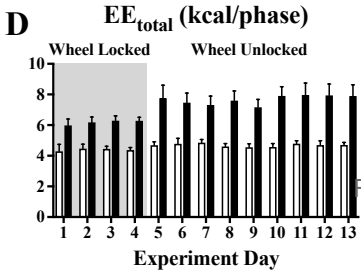
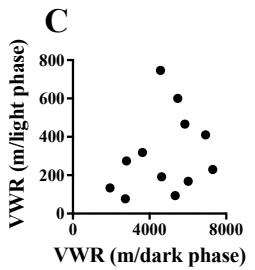
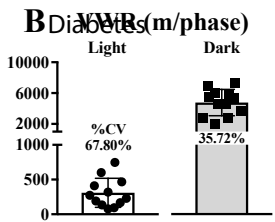
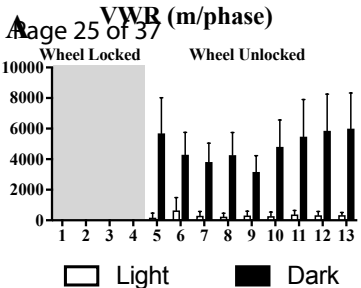
Figure 6: Reduced duration of episodic inactivity following wheel access predicts voluntary wheel running distance. Behavior frequency was determined for 12 consecutive hours during the last dark phase with the wheel locked or unlocked (A). The response of individual mice to an unlocked wheel (unlocked / locked) was analyzed using two-dimensional hierarchical clustering (mouse: x-axis, behavior: y-axis) and visualized as a heat map (B). The average speed of long or short roaming was measured in mice with a locked or unlocked wheel (C). The total distance traveled during short or long roaming was determined in mice with a locked or unlocked wheel (D). Time spent short roaming (open circles; dashed line) when the wheel was unlocked was positively associated with VWR (E). The response in short roaming to the wheel being unlocked revealed an even stronger positive association with VWR (F). N=12 mice. * indicates $p < 0.05$ between groups using paired Student's t-test.

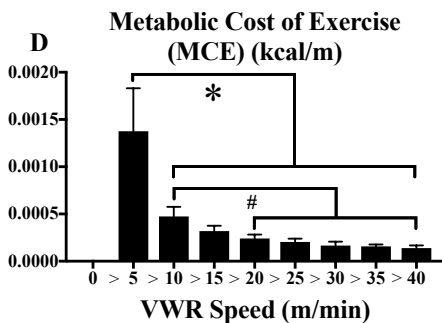
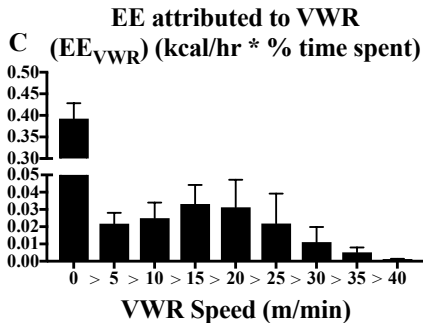
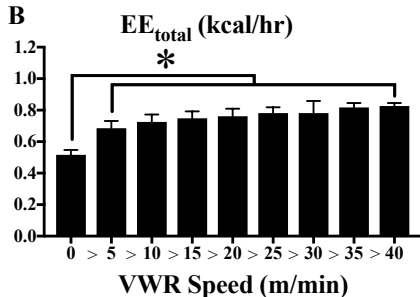
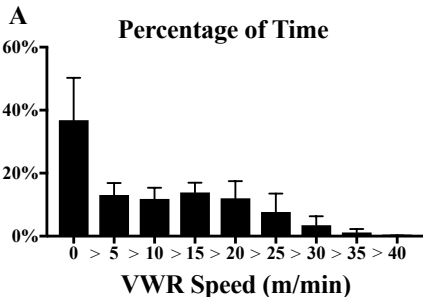
Figure 7: Duration and rapid successive bouts predict voluntary wheel running distance.

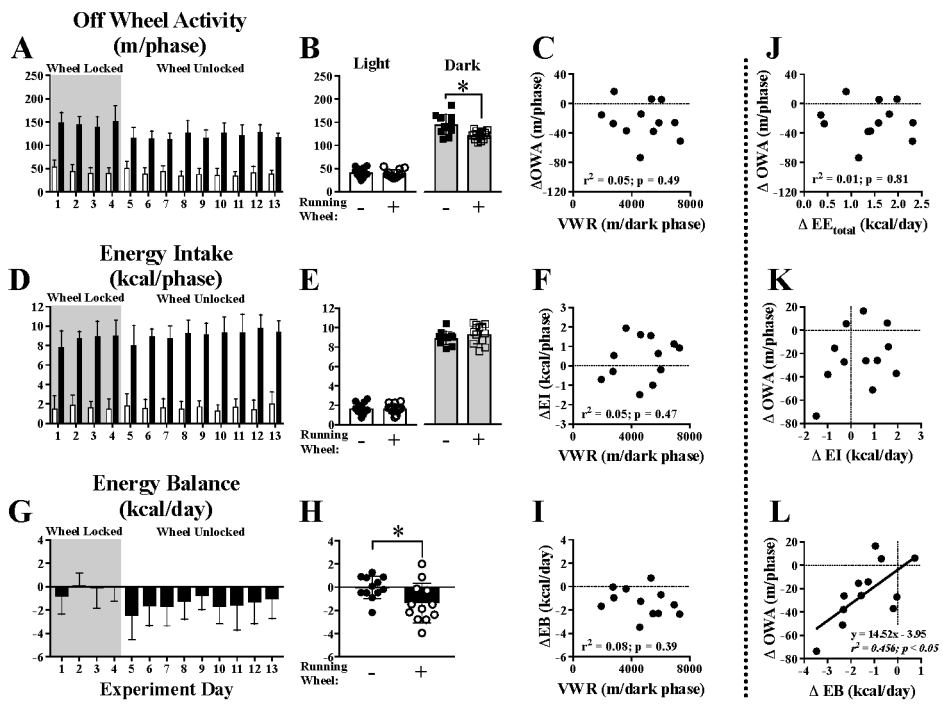
Mouse behavior transitions were continuously monitored for the last twenty-four hours with the wheel unlocked. The coupling of behaviors in sequence was mapped using a Markov chain plot that revealed “short roaming → VWR” and “VWR → short roaming” as prominent behavioral transitions (A). Lines represent group mean for N=12 mice.

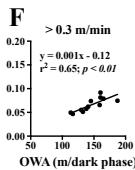
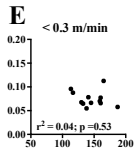
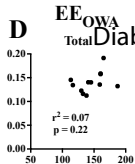
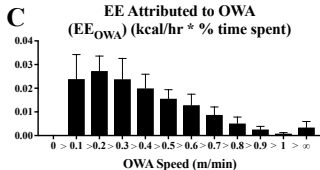
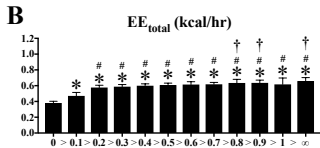
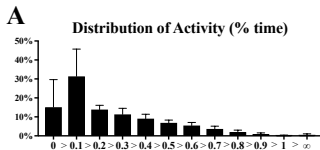
Figure 8: Reduced non-exercise physical activity preserves energy balance in response to

exercise. Top: In the absence of exercise, energy intake is matched with energy expenditure and resulting maintenance of body mass over time. Middle: Voluntary exercise increases energy expenditure that would be predicted to be additive for EE and thereby induce a pronounced negative energy balance. Bottom: The change in energy balance with voluntary exercise is less than predicted due to a reduction in non-exercise physical activity.

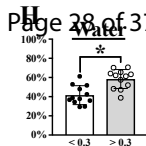
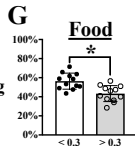




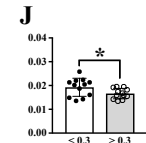
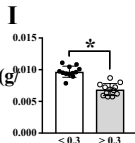




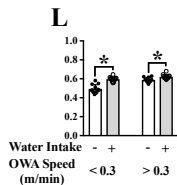
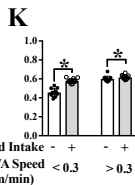
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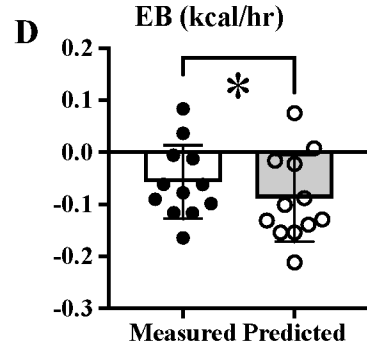
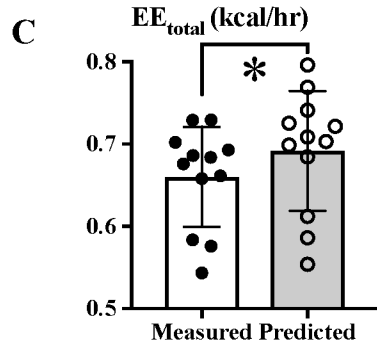
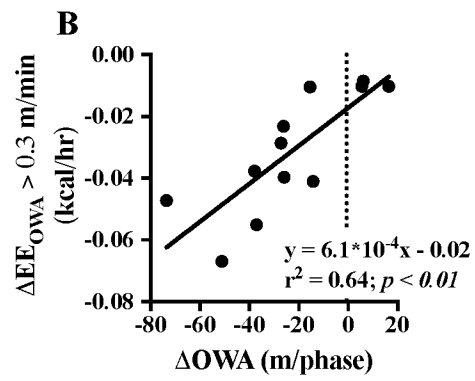
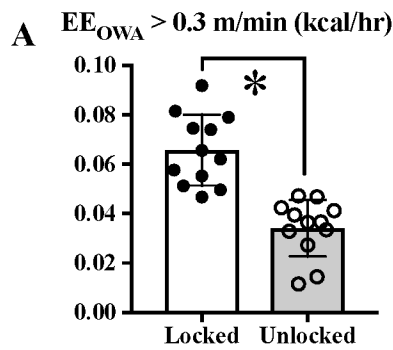


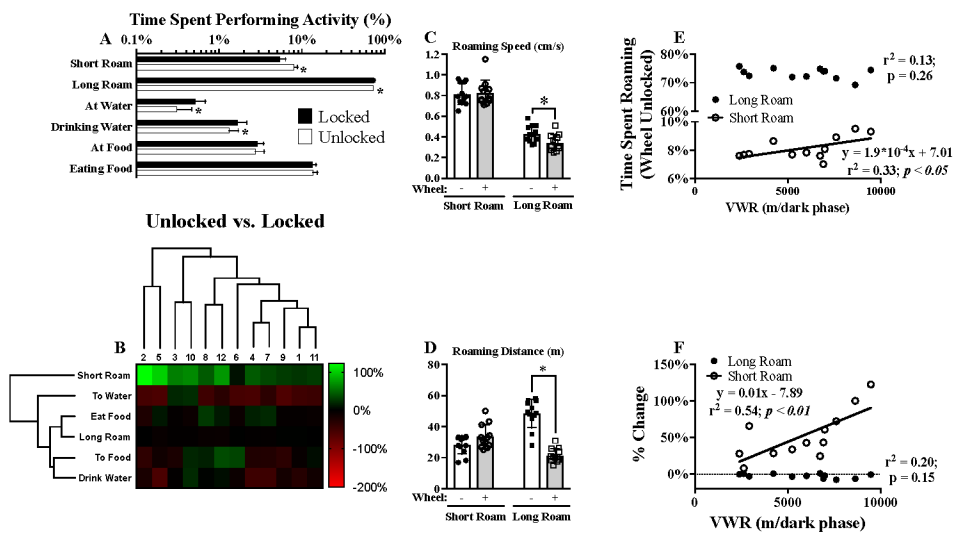
Rate of intake (g/min)



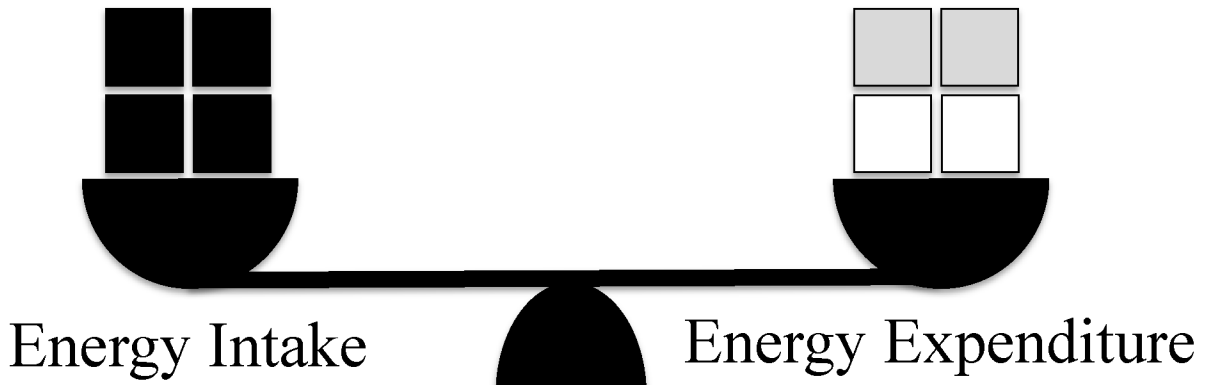
EE_{total} (kcal/hr)



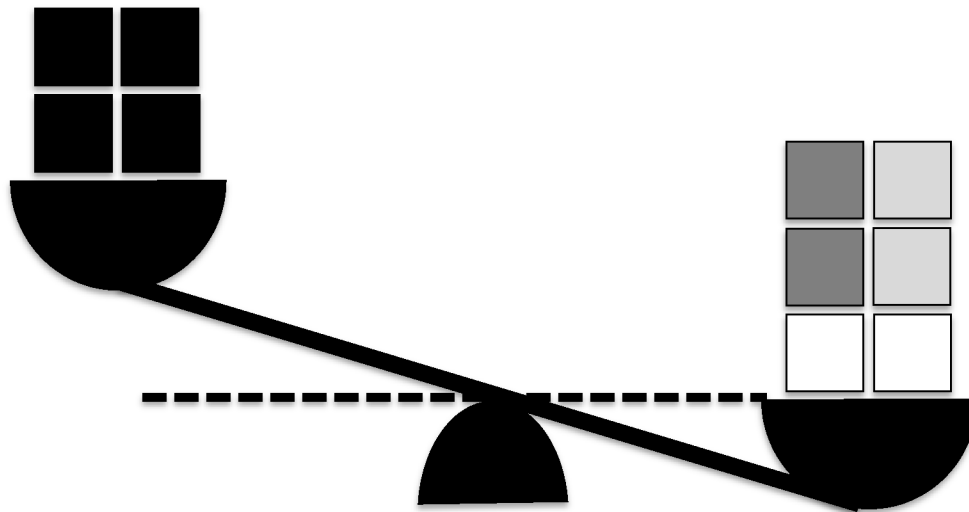




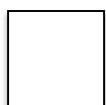
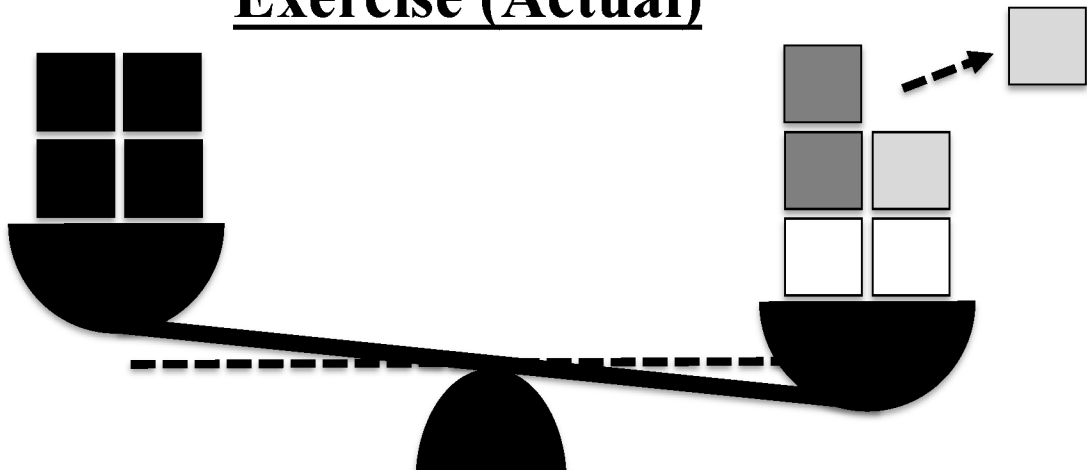
No Exercise



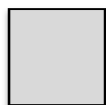
Exercise (Predicted)



Exercise (Actual)



Inactivity



Non-Exercise Activity



Exercise

SUPPLEMENTAL MATERIALS.

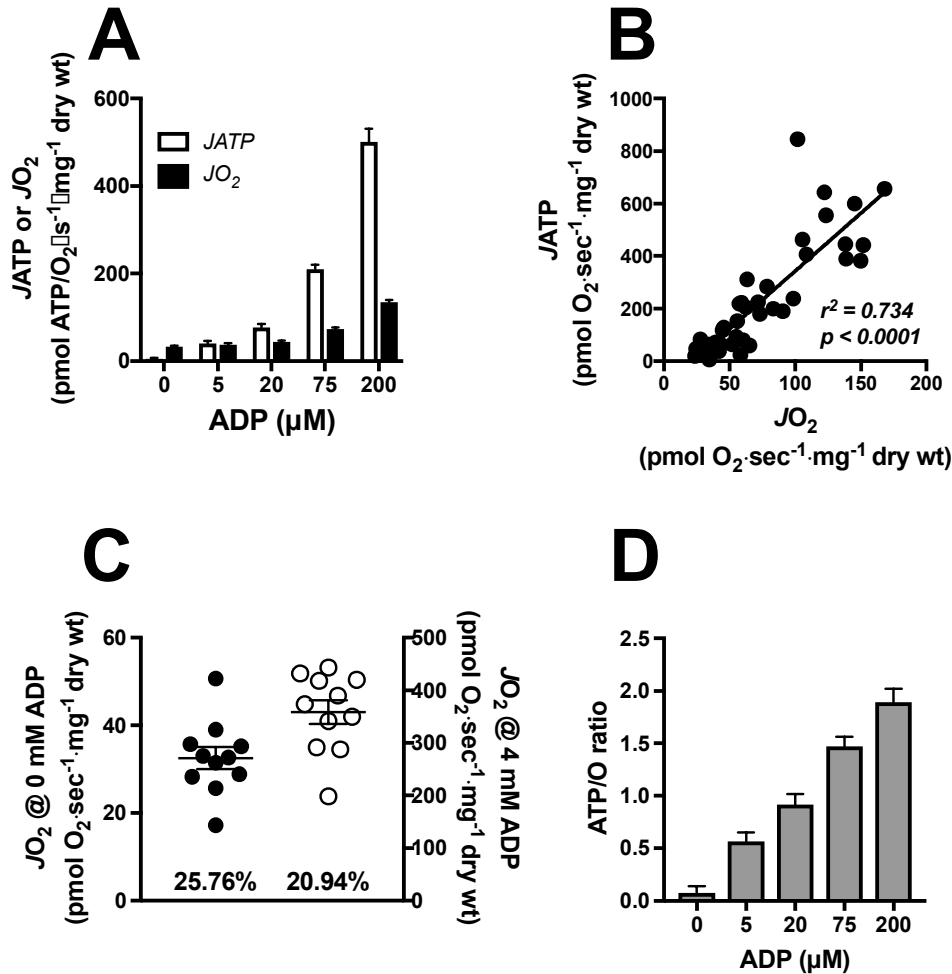
Indirect Calorimetry and Behavioral Mapping

A standard 12h light/dark cycle was maintained throughout the calorimetry studies. Mice were placed in metabolic cages located in the Vanderbilt Mouse Metabolic Phenotyping Center in a temperature- and humidity-controlled housing room. Energy expenditure (EE) and voluntary physical activity (OWA and VWR) measures were obtained using a computer controlled indirect calorimetry system (Promethion, Sable Systems, North Las Vegas, NV). The metabolic cages consist of the home cage with bedding, each equipped with water bottles and food hoppers connected to load cells for food and water intake monitoring. All animals had *ad libitum* access to standard rodent chow and water throughout the study. The air within the cages was sampled through micro-perforated stainless steel sampling tubes located in the inner bottom rim of the cages to ensure that the cage air is sampled uniformly, and that the temperature inside the cages does not exceed the temperature within the room. Respiratory gases are measured with an integrated fuel cell oxygen analyzer, spectrophotometric CO₂ analyzer, capacitive water vapor partial pressure analyzer and barometric pressure analyzer (GA3, Sable Systems, Las Vegas, NV). The system uses two GA-3 analyzers operating in parallel, devoted to eight cages apiece, to maximize throughput. Gas sensors were calibrated with 100% N₂ as zero reference and with a span gas containing known concentrations of CO₂. The gain of the O₂ channel was adjusted at each incurrent measurement so that incurrent O₂, after correction for water vapor dilution and barometric pressure, yields a concentration of 20.94% STPD (standard temperature and pressure, dry). The Promethion Metabolic Analyzer utilizes a pull-mode, negative pressure system. Two multi-channel mass-flow

generators measure and control air flows (FR8, Sable Systems, Las Vegas, NV). The ex-current flow rate was set at 2000 mL/min. Water vapor was continuously measured and its dilution effect on O₂ and CO₂ mathematically compensated for in the analysis stream (17). O₂ consumption and CO₂ production were measured for each mouse at 5 min intervals for 30 seconds. Incurrent ambient air reference values were determined after measuring 4 cages. Respiratory quotient (RQ) was calculated as the ratio of CO₂ production to O₂ consumption. Energy expenditure was calculated using the Weir equation: $\text{Kcal/hr} = 60 * (0.003941 * \text{VO}_2 + 0.001106 * \text{VCO}_2)$ (18). OWA was defined as ambulatory activity that was determined every second. Ambulatory activity and position were detected with XYZ beam arrays with a beam spacing of 1.0 cm interpolated to a centroid resolution of 0.25 cm. Consecutive adjacent infrared beam breaks are counted and converted to distance, with a minimum movement threshold set at 1 cm. VWR was also measured continuously as revolutions of a 36.12 cm circumference running wheel converted to distance. Data acquisition and instrument control were coordinated by MetaScreen and the raw data were processed using ExpeData (Sable Systems, Las Vegas, NV) using an analysis script detailing all aspects of data transformation.

Each cage in the Promethion metabolic phenotyping system was also equipped with an XYZ beam break array (BXYZ-R, Sable Systems, Las Vegas, NV) (physical light fence spacing 1.0 cm, interpolated 2.5 mm), a voluntary running wheel (36.12 cm circumference), and mass measurement modules (resolution 2 mg) for food intake, water intake, and body mass measurement, the latter in conjunction with an enrichment habitat

that the mouse could voluntarily enter or leave. Raw measurements from all sensors in all cages in the system (in conjunction with other measurements such as temperature, flow rates, gas concentrations etc.) were recorded every second. As a result, the position and activity of each animal in the system could be determined simultaneously with one-second temporal resolution. This “EthoScan” is equivalent to focal animal behavior sampling (Altmann, 1974), but for all individual animals in the experiment simultaneously. For this experiment, nine different behaviors were unambiguously distinguished when the running wheel was locked or unlocked. These activities were defined as: food intake (Eat Food), interacting with the food hopper but without food intake (To Food); water intake (Drink Water), interacting with the water dispenser but without water intake (To Water); running on the running wheel (Wheel), entering the body mass habitat (In Home); interacting with the body mass habitat without entering it (At Home); less than 60 seconds where no cage sensors are triggered (except for the XYZ beam break array)(Short Roam), greater than 60 seconds where no cage sensors are triggered (except for the XYZ beam break array)(Long Roam). In the case of intake behavior, intake was distinguished from hopper or dispenser interaction by a reduction in the hopper or dispenser mass, as determined by Student’s *t*-test applied to mass measurements before and after each behavior. The threshold for mass significance was set at $P \leq 0.05$, and allowed confident measurement of intake events down to 2 mg.



Supplementary Materials – Figure 1: Mitochondrial energetics measurements.

Rates of ATP production (J_{ATP}) and O₂ consumption (J_{O_2}) were simultaneously measured in permeabilized red gastrocnemius following a four-hour fast (A). J_{ATP} and J_{O_2} were highly related across the range of [ADP] added (B). Average values for J_{O_2} prior to the addition of ADP and at maximal ADP-stimulated respiration ([ADP] = 4 mM) are shown in (C). ATP/O ratio ($ATP/O = J_{ATP} / (J_{O_2} * 2)$) increased as a function of the [ADP] added (D). N=12 mice; assays were performed in duplicate and the average value reported for each mouse.

		Subsequent Behavior								
		Eat Food	To Food	Drink Water	To Water	Wheel	In Home	To Home	Long Roam	Short Roam
Initial Behavior	Eat Food	0	0	17.86	3.39	1.35	0.14	0.11	13.78	63.37
	To Food	0	0	7.05	4.18	6.95	0.08	0.22	15.26	66.26
	Drink Water	4.3	4.85	0	0	1.56	0.08	0.04	20.39	68.78
	To Water	12.36	19.92	0	0	2.14	0.56	0.09	11.41	53.52
	Wheel	0.61	3.57	0.66	0.14	0	0.01	0.03	8.96	86.01
	In Home	0.12	0.04	0.03	0.24	0	0	0	15.57	84
	To Home	0.53	1.04	0	0.15	0.37	0	0	15.05	82.85
	Long Roam	20.5	28.55	12.45	2.58	27.08	4.13	4.7	0	0
	Short Roam	15.42	17.67	15.74	2.85	39.03	4.88	4.4	0	0

Supplemental Materials – Table 1: Behavioral transition probability matrix for mice with an unlocked running wheel.