Physical activity: benefit or weakness in metabolic adaptations in a mouse model of chronic food restriction?

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Méquignon M, Caron E, Zgheib S, Steienard A, Zizzari P, Tolle V, Cortet B, Lucas S, Prévot V, Chauveau C, Viltart O. Physical activity: benefit or weakness in metabolic adaptations in a mouse model of chronic food restriction? Am J Physiol Endocrinol Metab 308: E241–E255, 2015. First published December 2, 2014; doi:10.1152/ajpendo.00340.2014.—In restrictive-type anorexia nervosa (AN) patients, physical activity is usually associated with food restriction, but its physiological consequences remain poorly characterized. In female mice, we evaluated the impact of voluntary physical activity with/without chronic food restriction on metabolic and endocrine parameters that might contribute to AN. In this protocol, FRW mice (i.e., food restriction with running wheel) reached a crucial point of body weight loss (especially fat mass) faster than FR mice (i.e., food restriction only). However, in contrast to FR mice, their body weight stabilized, demonstrating a protective effect of a moderate, regular physical activity. Exercise delayed meal initiation and duration. FRW mice displayed food anticipatory activity compared with FR mice, which was strongly diminished with the prolongation of the protocol. The long-term nature of the protocol enabled assessment of bone parameters similar to those observed in AN patients. Both restricted groups adapted their energy metabolism differentially in the short and long term, with less fat oxidation in FRW mice and a preferential use of glucose to compensate for the chronic energy imbalance. Finally, like restrictive AN patients, FRW mice exhibited low leptin levels, high plasma concentrations of corticosterone and ghrelin, and a disruption of the estrous cycle. In conclusion, our model suggests that physical activity has beneficial effects on the adaptation to the severe condition of food restriction despite the absence of any protective effect on lean and bone mass.

food restriction; physical activity; animal model; physiological adaptation; anorexia nervosa

Physical activity is known to have beneficial and protective effects for health exerted at both the peripheral and central levels in mammals. Indeed, exercise reduces the prevalence of obesity and its comorbidities like type 2 diabetes, cardiovascular pathologies, and hepatic steatosis but also stress, anxiety, and depression (16, 58, 69). However, excessive physical activity could lead to injuries (20), especially when combined with restrictive diet (38). Among the various alterations observed in some categories of athletes, growth retardation, muscular atrophy, amenorrhea or metabolic disturbances, and susceptibility to eating disorders might have deleterious consequences on health (52, 61, 64, 65). These high-risk conducts are also observed in restrictive anorexia nervosa (AN) with 35–80% of patients who present excessive physical activity associated with a severe chronic food restriction (14, 15), even if this last parameter has been omitted from the diagnostic criteria currently used (1, 2). Beside severe weight loss and metabolic disturbances, AN is also associated with endocrine alterations, reproductive dysfunctions, osteopenia, and osteoporosis (24, 29, 30, 40, 41, 42, 43, 47). The prevalence of AN has increased drastically within recent decades and is currently the third-largest cause of chronic illness in teenagers (35, 44). It leads to poorly known central and/or peripheral reprogramming that permits the individual/organism to adapt to a chronically reduced energy supply. The role of enhanced activity in this reprogramming remains to be determined.

It remains an open question whether the physical activity observed in AN is beneficial or deleterious for health and whether it forms an adaptation to this drastic caloric restriction. Answering this question would inform clinicians as to whether it is better to promote regular physical activity instead of existing hyperactivity or inactivity in AN patients. For this purpose, the use of animal models mimicking distinct physiological components of AN is essential. A number of different animal models have been developed, especially environmental models, and one of these associates wheel running access with time-restricted feeding. This model was inappropriately called “self-starvation” or, more recently, “activity-based anorexia” (ABA) and was developed in the rat by Routtenberg and Kuznesof (56). Many symptoms described in AN were reproduced in this model, including increased activity and various physiological alterations (33, 34, 70). In the ABA paradigm, the phenotype observed can be amplified or reduced depending on the rodent strain, sex, and age (28, 39). However, most of the studies were performed with male rodents, whereas the majority of AN cases are described in female patients. Regarding self-starvation as part of a cognitive/emotional aspect of the disease proved difficult, even impossible, to mimic in mice. Indeed, in the ABA model, the self-starvation, which is not always observed (36), might be related to physiological adaptations to maintain thermogenesis and to compensate for the dryness of the pellets since the presentation of humidified pellets during the 2-h time-restricted feeding or an increasing of the room temperature led to the disappearance of the
voluntary food restriction (7, 8). These data support more adaptations to physiological changes than a cognitive drive to reduce feeding. Moreover, adopting this kind of “self-starvation behavior” caused the mice to die rapidly (usually 5–7 days) due to important energy deficit and weight loss. This constituted another drawback of the ABA model since we expected to study the evolution of physiological parameters in the long term.

The present study aimed to circumvent these drawbacks. Because self-starvation is not necessary to study physiological consequences of activity associated with food restriction, we have changed the initial protocol by working on female mice and by limiting the amount of food distributed (50%) without time limitation. The female mice of the “activity” groups had free access to wheel running. Activity added to food restriction allowed fast and severe body weight loss associated with protocol durations as long as 8 wk. Our data show that when food restriction is associated with physical activity, it results in a better metabolic adaptation, although it accelerates weight loss in the short term compared with pair-fed mice without wheel access. Our results provide new insights regarding the potential mechanisms that lead to the physiological deregulations observed in AN patients with or without hyperactivity.

METHODS

Animals and procedures. Adult C57BL/6J female mice (7 wk old, average initial body weight 18.3 ± 0.1 g; Charles River Laboratories, L’Arbresle, France) were housed two per cage to avoid isolation stress effects until the end of the protocol. They were kept in a pathogen-free barrier facility maintained at 21.5°C with a 12:12-h dark-light cycle (lights on at 0730). During 1 wk of acclimation, mice were weighed every day to get used to handling and had free access to water and standard chow diet (4.30% fat, 22.30% protein, and 51.20% carbohydrate; Special Diet Service RM3; Dietex, Essex, UK). The mice were then divided into four groups. In the experimental group “food restriction and wheel” (group FRW), mice were placed in a cage equipped with a free running wheel (Intellibio, Seichamps, France) and submitted to a quantitative food restriction comprising 30%/day for 3 days and then 50%/day until the end of protocol. This restriction was calculated from the total food eaten in a cage full of mice fed ad libitum (group AL) the day before to have a standard quantity of food eaten per day. We also used two other control groups: a pair-fed group [group FR (food restricted)] and mice fed ad libitum but in cages equipped with a wheel (group ALW). The presence of two mice in the home cage did not permit to measure separately the quantity of food ingested by each animal. However, the use of metabolic cages (see below) where mice were studied individually has permitted us to validate the data obtained and to precisely know the food intake pattern of the four groups of mice. Body weight and food intake (distribution at 1830) were monitored daily in the short-term (15 days) and long-term (≤55 days) protocols (Figs. 1 and 2). Control of body weight was done to ensure that two mice in the same cage had similar feeding behavior and activity. If evident differences were noted between the two mice, the data obtained from this cage were excluded from analysis. At different times in the protocol (Fig. 1), some subgroups of mice were isolated for 2–3 days in metabolic cages (TSE Systems, Hamburg, Germany) to monitor ambulatory activity, food intake pattern, O₂ and CO₂ consumptions, and energy expenditure. The cages of FRW and ALW mice were equipped with a wheel (178 mm in diameter) to maintain their physical activity without totally modifying the paradigm. The number of mice that have been used varied between six and 24 per group, depending on the experiments and the protocols considered. The variation in the number of animals was due only to specific experiments done to obtain the metabolic data or blood samplings performed at different stages of the protocols. Such manipulation might change slightly and transiently the behavior of the mice (feeding or activity). All measurements related to the metabolic behavior done in metabolic cages were made on mice that followed the previously described feeding protocol, since we wanted to evaluate the effect of this protocol on various metabolic parameters. However, the experiments related to blood samples and tissues collection were done on mice that had received the same amount of food (~3.5 g/cage) at the same hour the day before euthanization/samplings to maintain all mice in similar fasted conditions and minimize intragroup variations. No mice died in any protocol. Further details are given in the legends. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and approved by the Regional Ethics Committee of Nord-Pas de Calais of Lille, France (protocol no. CEEA 392012).

Activity monitoring. The daily locomotor activity of ALW and FRW mice was assessed in their home cage equipped with a wheel (diameter: 230 mm; width: 50 mm; 1 revolution = 0.72 m) and linked to a computer system that measured interval counts (10 min) per mean wheel revolution (ActiviWheel Software; Intellibio, Seichamps, France). When the mice were placed individually in the metabolic cages (Fig. 1), the total ambulatory activity of all mice was evaluated with an ambulatory captor (infrared light-beam frame ActiMot2) that measured activity in three dimensions (x-, y-, and z- axes).

Reproductive functions. The estrous cycle was assessed by vaginal smears performed before food distribution and at different stages of the procedure: between day 5 before the beginning of the protocol and day 16 for the short-term protocol and between days 48 and 55 for the long-term protocol. We placed the tip of the pipette filled with saline solution (10 μl of NaCl, 9 g/l) 5 mm into the vagina, flushed the vagina about five times, and put the final collection containing the vaginal secretion on a glass slide. We observed the cells without coloration under a light microscope (Leica Biosystems, Wetzelar, Germany) with a final magnification of ×100 for immediate estrous cycle stage determination (estrus, diestrus, and metestrus). Proestrus stage that corresponds to short transient stages was not determined. The day of euthanization and days 13 and 55 after estrous cycle determination, left and right ovaries were collected, and the uterus was weighed. Ovaries were then fixed in Bouin fixative and processed through graded alcohol until paraffin wax embedding. Paraffin-embedded ovaries were serially sectioned at 5-μm thickness with a microtome Leica Reichert-Jung (Leica Biosystems, Nussloch, Germany) and stained with hematoxylin-eosin. Observations and photos were made using a Leica microscope (Leica Microsystems, Wetzelar, Germany) equipped with a camera. The size of the ovaries was measured following two axes (width and length) with ImageJ software (http://imagej.nih.gov/ij/) from slices containing the largest ovary section.

Body composition. Body composition was determined at different time points during the protocols (D-1/D15/D55; Fig. 1) using an “in vivo μCT scanner for small laboratory animals” (LaTheta LCT-100; Hitachi Aloka Medical, Tokyo, Japan) to evaluate bone, lean, and fat

Fig. 1. Study design for the short- and long-term protocols. Arrows indicate X-ray CT scan sessions; double arrows correspond to intraperitoneal glucose tolerance test, and gray squares show the metabolic cage sessions. D, days.
masses. Mice were anesthetized by intraperitoneal injection of ketamine and xylazine mix. About 60 CT slices per mouse were made at 500-µm intervals between shoulders and the posterior legs. Slices were analyzed by Aloka software, and mouse head and tail were excluded from analysis for bone [bone mineral content (BMC)], fat (visceral and subcutaneous), and lean mass measurements.

Metabolism and energy expenditure monitoring. The data were obtained in metabolic cages (LabMaster-Calosys-Calorimetry System; TSE Systems) for a period of 3 days (Fig. 1). Food and water intake patterns were measured every minute by sensing units, and the values were added to present cumulative data. The system measured the volume of O₂ consumed and the volume of CO₂ produced over a 15-min period four times per hour. These values were averaged to determine the rate of CO₂ produced (VCO₂) and oxygen consumed (VO₂) in milliliters/hour. We determined respiratory exchange ratio (RER), the energy expenditure (EE), and the fatty acid (FA) oxidation using different equations (11, 45, 46): 

\[
\text{RER} = \frac{V_{CO2}}{V_{O2}}; \quad \text{EE} = \frac{\text{FA oxidation (kcal/h)}}{\text{EE} \times [1 - \text{RER}]/0.3}
\]

The RER is an indicator of the proportion of fat and carbohydrate oxidation under specified conditions (46) and ranges between 0.7 (complete reliance on fat oxidation) and 1.0 (complete reliance on carbohydrate oxidation).

Glycemia and intraperitoneal glucose tolerance testing. Glycemia was measured (OneTouch Vita; Lifescan, Milpitas, CA) in the morning (between 0800 and 1000) after the night-feeding period at different stages (D15 and D50). To assess glucose tolerance, we performed
an intraperitoneal glucose tolerance test (IPGTT). All mice received the same quantity of food the day before the experiment (at 1600) to maintain all mice in a similar satiated condition. Then, nonsedated mice were weighed and injected with a glucose solution (1.5 g/kg). Their glyceremia were assayed from blood sample drops taken from the tail at 0, 5, 15, 30, 60, 90, and 120 min postinjection.

Euthanization and tissue collection. At the end of the short- and long-term protocols, mice were euthanized. All mice received the same amount of food (~3.5 g/cage) the day before euthanization to maintain all mice in similar fasted conditions and minimize intragroup assay variations. They were deeply anesthetized in the morning with an overdose of ketamine (100 mg/kg) and xylazine mix (20 mg/kg).

Blood was collected through cardiac puncture with a 1-ml syringe and kept at 4°C for ~2 h in neutral tubes, except for ghrelin, which was due to the low stability of this hormone until centrifugation (8,000 rpm for 10 min, 4°C. Centrifuge 5414 R; Eppendorf, Hamburg, Germany). Plasma aliquots were then deeply frozen in liquid nitrogen and stored (~80°C) until they were assayed. Mice were then dissected to weigh liver and gastrocnemius muscle. The largest liver lobe was collected and frozen in liquid nitrogen until glycogen liver assay.

Measurement of hepatic glycogen. About 100 mg of the largest liver lobe of each mouse was thawed and homogenized in 0.03 M HCl. Then, 400 μl of 1.25 N HCl was added to 100 μl of homogenate and was mixed and heated for 1 h at ~75°C. The samples were centrifuged at 14,000 rpm for 30 min. Ten microliters of supernatant was mixed with 1 ml of glucose oxidase reagent (Sigma-Aldrich, St. Louis, MO). After 10 min of incubation at 37°C, the reaction was stopped by adding 200 μl of 12 N H2SO4. Absorbance was read at 505 nm. Glycogen type III from rabbit was used to make the standard curve (Sigma-Aldrich).

Blood assays. All the samples were analyzed in duplicate. Plasma leptin was measured using an ELISA kit (R & D Systems Quantikine Europe, Abingdon, UK). Intra- and interassay coefficients of variations were 4.4 and <4.7%, respectively. Plasma corticosterone was measured using the AssayMax Corticosterone ELISA Kit (AssaysPro, St. Charles, MO). Intra- and interassay coefficients of variations were <4.8 and <7.3%, respectively. Plasma acyl and des-acyl ghrelin concentrations were evaluated by specific EIA (A05118 for the acylated form and A05117 for the unacylated form; SPIbio Bertin Pharma, Montigny le Bretonneux, France). Blood samples were collected in tubes containing EDTA (1 mg/ml final) and PHMB (0.4 mM final), a serine protease inhibitor, and then centrifuged (8,000 rpm for 10 min, 4°C Centrifuge 5414 R; Eppendorf, Hamburg, Germany) to sample plasma that was then acidified with HCl (0.1 N final) to preserve acylation. Plasma nonesterified fatty acids (NEFA) were measured by an enzymatic Wako kit (Stanbio Laboratory), and plasma triglycerides and plasma β-hydroxybutyrate (ketone bodies) were analyzed using an enzymatic Stanbio kit (BDS International Diagnostics, Schwetzingen, Germany).

Statistical analysis. All results are expressed as means ± SE, and the statistical analysis was performed using Statistica software (StatSoft). Graphs were generated using GraphPad Prism 5.01 (Abacus Concepts, Berkeley, CA). Analysis of normality and equality of variances were tested to select the adequate statistical test. Two-way ANOVA followed by a Bonferroni post hoc test or a nonparametric ANOVA followed by Tukey post hoc test was used when appropriate. A two-way ANOVA for repeated measurements was performed to analyze the time course for metabolic data and IPGTT. The areas under the curve (AUC) were calculated by the trapezoidal method. Significance was set at a P < 0.05, and only interaction effects are indicated in the figures.

RESULTS

Physical activity transiently increased food restriction-induced body weight loss. The first and simplest criterion to determine the effect of activity added to food restriction is the body weight gain. Here, we showed that by day 15 (D15) of food restriction there was a significant weight loss in the FR and FRW groups compared with the AL and ALW ad libitum groups (interaction of food × activity, F1–24 = 18.17, P < 0.001; Fig. 2, A and B). This decrease was significantly more drastic in FRW than FR mice from D6 to D22 (P < 0.05). From D42 to D55, statistical analysis revealed an effect of activity (F1–24 = 6.66, P < 0.05) and food restriction (F1–24 = 1.243.05, P < 0.001) on body weight loss but no interaction between these two parameters. Surprisingly, after 35 days a slight but significant regain of body weight was observed in FRW mice (P < 0.001, from D43 to D55), leading to significant differences between body weight of FRW and FR groups. (P < 0.001 at D55; Fig. 2A).

Physical activity also impacted the food intake pattern (Fig. 2, C and D). On D15, FRW mice ate their pellets more slowly than FR mice along the first 2 h after lights off (interaction of food × activity, F1–23 = 4.71, P < 0.05). FR and FRW mice finished their meal within 3 and 4 h after food distribution, i.e., at 2130 and 2230, respectively. AL and ALW mice ate slowly but continuously until the end of the dark phase at 0730. On D55, FR and FRW adopted the same pattern of feeding (interaction of food × activity, F1–22 = 4.15, P = 0.054; Fig. 1), but they ate all of the food within 1.5 h only. Each group showed a constant water intake pattern throughout the protocol, with FR and FRW mice presenting faster and higher water consumption than AL and ALW mice during the first part of the night, i.e., when they were eating (Fig. 2, E and F). Thus, during the first weeks of food restriction, physical activity slowed down the food intake. Later, FR and FRW mice presented the same pattern of food intake, with a faster feeding rate than before. Surprisingly, after 35–40 days of protocol, a slight but significant increase in body weight appeared only in FRW mice (Fig. 2A). Factors potentially involved in the early and fast body weight decrease and in the late body weight increase shown in FRW mice were studied in subsequent experiments.

Decrease in physical activity appeared with body weight regain in food-restricted active mice. FRW mice presented a biphasic pattern of daily physical activity during the long-term protocol observed in both home cage (Fig. 3) and metabolic cages (Fig. 4). From D0 to D35, FRW mice displayed a wheel-running activity that was similar to that of ALW mice (Fig. 3). Then and until the end of the protocol, FRW mice showed a significant lower daily activity than ALW mice (P < 0.05; Figs. 3 and 4D). These results were confirmed by 24-h locomotor activity measured in metabolic cages (Fig. 4), which detailed precise information about the nightly and daily physical activity patterns in all groups. At D15, FRW mice displayed the highest activity compared with the other groups during the day (interaction of food × activity, F1–36 = 13.24, P < 0.01; Fig. 4, B and C), especially from 1200 to 1900 (interaction of food × activity, F1–36 = 10.83, P < 0.01; Fig. 4C). This phenomenon, which resonates with the increase in physical activity seen in AN before feeding time (60), is described in the literature as food anticipatory activity (FAA), which began several hours before food distribution. Such activity observed before feeding might be an association of foraging and FAA. Contrary to FRW mice, FR mice did not exhibit FAA (Fig. 4C). During the dark period, ALW mice presented a constant and higher ambulatory activity compared.
with AL and FRW mice (interaction of food × activity, \( F_{1,36} = 5.17, \ P < 0.05 \); Fig. 4, B and C). On D45, surprisingly, in FRW group, the FAA measured on D15 in metabolic cages disappeared (\( P < 0.001 \); Fig. 4, E and F). The persistent activity observed concerned more of the activity devoted to food intake as it appeared at the time the food was distributed (Fig. 4F). The disappearance of the FAA was responsible for the observed decrease in daily activity in FRW mice (Fig. 4D). FRW and ALW mice continued to exhibit the highest ambulatory activity during the dark period compared with their respective controls (interaction of food × activity, \( F_{1,26} = 5.15, \ P < 0.05 \); Fig. 4F). These data demonstrate that mice used to running on a wheel (ALW and FRW) are more active than mice that are not used to running (AL and FR). Moreover, FAA is shown only in FRW and not in FR mice. When the protocol is extended, the FRW mice’s daily activity decreased mainly because of the disappearance of FAA. This decrease of activity that coincided with the body weight regain was observed only in FRW mice.

**Food restriction induced alterations in reproductive function.** Activity, when it becomes excessive, leads to loss of estrous cycle in restrictive AN patients as well as in athletes. In our study, we showed that food restriction by itself associated or not with physical activity rapidly induced a disruption of the estrous cycle (Fig. 5) associated with a closure of the vaginal opening and a decrease in uterus mass (Table 1) observed on D15 and D55. The sizes of the ovaries of both FR and FRW mice were significantly reduced compared with AL and ALW mice (Table 1). Thus, physical activity did not exacerbate the impact of reduction of feeding in the reproduction function.

**Physical activity precipitated early alterations of fat mass in food-restricted mice.** Besides loss of fat, AN patients also show muscular atrophy and osteopenia/osteoporosis in the long term (41), for which the impact/role of physical activity is poorly understood. To this end, in the present study, we explored fat, lean, and bone mineral mass changes. The different measurements obtained at different times of the long-term protocol (Fig. 1) with the X-ray CT scanner (Fig. 6) showed more precisely the impact of physical activity associated or not with food restriction on body composition. On D15, food restriction induced a 19% lower lean mass in FR and FRW mice compared with AL and ALW mice (food effect, \( F_{1,20} = 132.02, \)

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**Fig. 3.** Effects of food restriction on wheel running activity in the home cages for ALW and FRW groups. The mean cumulative wheel revolution was calculated for ALW and FRW groups (\( n = 2/cage \)) throughout the protocol. Rectangles indicate the metabolic cage sessions (see Fig. 4) to measure ambulatory activity for all groups (\( n = 1/cage \)). ALW, \( n = 6 \); FRW, \( n = 14 \). \#\( P < 0.05 \), FRW vs. ALW. Significance was set following 2-way ANOVA.

**Fig. 4.** Effects of food restriction on locomotor activity in the metabolic cages for the 4 groups. A and D: total activity was analyzed throughout the day on D15 (A) and D45 (D) and averaged for the dark (active phase) and light periods. B–E: measurements were taken at different periods of the protocol: short term (B and C) and long term (D and E). The locomotor activity was measured continuously and averaged every hour in C (short term) and F (long term). Values are expressed as means ± SE. AL, \( n = 8 \); ALW, \( n = 10 \); FR, \( n = 10 \); FRW, \( n = 12 \) for the short-term and \( n = 10 \) for the long-term protocols. \*\( P < 0.05 \), FR or ALW vs. AL; \#\( P < 0.05 \), FRW vs. ALW; §\( P < 0.05 \), FR vs. FRW. Significance was set following 2-way ANOVA.
Fig. 5. Impact of food restriction associated or not with physical activity on estrous cycles. One example of mouse from each group was given along the protocol, representing the whole group. AL, \( n = 6 \); ALW, \( n = 6 \); FR, \( n = 6 \); FRW, \( n = 6 \). D, diestrus; M, metestrus; E, estrus; nd, nondetected.

The visceral fat mass was affected by both food restriction (food effect, \( F_{1,20} = 4.86, P < 0.05 \); Fig. 6B) and physical activity (activity effect, \( F_{1,20} = 26.57, P < 0.001 \); Fig. 5B). In particular, FRW mice exhibited a decrease of \(-87.20 \pm 5.04\%\) compared with FR mice fat mass. Interestingly, FR mice did not display such a drastic decrease \((-33.90 \pm 11.43\%)\) compared with the fat mass of AL mice. The visceral fat mass was affected by both food (\( F_{1,20} = 7.84, P < 0.05 \); Fig. 6G) and activity (\( F_{1,20} = 17.94, P < 0.001 \); Fig. 6C). The subcutaneous fat mass was affected only by activity (\( F_{1,20} = 30.72, P < 0.001 \); Fig. 6D).

On D55, the differences in lean mass did not change compared with D15 (food effect, \( F_{1,20} = 557.48, P < 0.001 \); Fig. 6A). The weight of the liver was affected by the restriction (food effect, \( F_{1,46} = 75.12, P < 0.001 \)), whereas the weight of the triceps was conditioned by both food and physical activity (food effect, \( F_{1,22} = 175.71, P < 0.001 \); activity effect, \( F_{1,22} = 6.31, P < 0.05 \) (Table 1). For the fat mass, an interaction of food \( \times \) activity was noted (\( F_{1,20} = 9.76, P < 0.005 \); Fig. 6B), with a pronounced decrease for FRW and FR mice compared with ALW and AL mice \((P < 0.005)\) and ALW to AL mice, respectively \((P < 0.005)\). Similar comparisons were obtained for visceral fat mass (interaction of food \( \times \) activity, \( F_{1,20} = 7.91, P < 0.05 \); Fig. 6C) and subcutaneous fat mass (interaction of food \( \times \) activity, \( F_{1,20} = 10.09, P < 0.05 \); Fig. 6D). FR mice presented a higher decrease in fat tissue compared with AL mice \((-88.03 \pm 2.59\%)\) than FRW mice compared with ALW and FR mice, respectively \((-67.88 \pm 7.76\% \text{ and } +70.8 \pm 40.45\%, P < 0.05)\). Finally, the bone mass acquisition might be stopped between D15 and D55 in FR and FRW mice. Indeed, restriction did not permit a gain of BMC (food effect, \( F_{1,20} = 43.95, P < 0.001 \); Fig. 6E) for the FRW and FR groups compared with ALW and AL groups. As expected, ALW mice exhibited a trend toward a higher BMC than AL mice. Collectively, these data suggest that physical activity accelerates the effects of food restriction on fat tissue without protecting muscle and bone mass.

Physical activity associated with food restriction induced an adaptation in energy metabolism. Long-term food restriction associated or not with physical activity has been suggested to induce changes in the energy metabolism, changes that are difficult to assess precisely in AN patients in the long term.

### Table 1. Alterations in metabolic and reproductive tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>AL</th>
<th>ALW</th>
<th>FR</th>
<th>FRW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver, mg</td>
<td>( n = 6 )</td>
<td>948.03 ± 30.81</td>
<td>958.55 ± 19.61</td>
<td>807.22 ± 18.91</td>
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<tr>
<td>Triceps, mg</td>
<td>( n = 6 )</td>
<td>108.13 ± 1.31</td>
<td>117.93 ± 1.38</td>
<td>87.12 ± 3.19</td>
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<td>Uterus, mg</td>
<td>( n = 6 )</td>
<td>100.23 ± 15.60</td>
<td>106.83 ± 18.15</td>
<td>21.18 ± 0.97</td>
</tr>
<tr>
<td>Ovary length, μm</td>
<td>( n = 6 )</td>
<td>1,658.92 ± 74.18</td>
<td>1,523.80 ± 63.99</td>
<td>1,397.23 ± 64.83</td>
</tr>
<tr>
<td>Ovary width, μm</td>
<td>( n = 6 )</td>
<td>1,177.83 ± 77.85</td>
<td>1,226.28 ± 84.26</td>
<td>988.69 ± 71.97</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; \( n = 6–14 \text{ group } \). D15, day 15; D55, day 55; AL, ad libitum; ALW, ad libitum and wheel; FR, food restriction; FRW, food restriction and wheel. Impact of food restriction associated or not with physical activity on the weight of liver, triceps, and uterus and on the ovaries’ size. All samplings were done in the morning (0830–1300). Significance was set following 2-way ANOVA. *Food effect; †activity effect.
(57). Animal models are thus useful to decipher metabolic adaptations that take place in response to long-term energy imbalance. On D15, EE was affected by both activity and food restriction during the dark period (interaction of food × activity, F1,36 = 15.13, P < 0.001; Fig. 7, A and B), with FR and FRW mice having a lower EE than their respective control AL and ALW mice (P < 0.001). During the day, similar differences were observed (food effect, F1,36 = 70.23, P < 0.001; Fig. 7, A and B). Over a period of 24 h, the pattern of EE for AL followed their ambulatory activity (Fig. 3). By contrast, FRW mice presented the same EE pattern as FR mice (6.15 ± 0.25 kcal/day in FR mice vs. 6.14 ± 0.20 kcal/day in FRW mice; data not shown), even if FRW mice displayed a higher physical activity level than FR mice (Fig. 4C). On D45, regarding the daily EE, FR and FRW mice had a lower EE than the ad libitum groups (interaction of food × activity, F1,26 = 18.41, P < 0.001; AL: 10.14 ± 0.13 kcal/day; ALW: 11.98 ± 0.29 kcal/day; FR: 5.96 ± 0.16 kcal/day; FRW: 6.05 ± 0.15 kcal/day). During the dark period, FRW mice showed a higher EE than FR mice but a lower EE than ALW mice (interaction of food × activity, F1,26 = 8.82, P < 0.01; Fig. 7, C and D) that might have been paralleled with their highest physical activity (Fig. 4F). However, despite a decrease in locomotor activity during the light period between D15 and D45, FRW mice displayed the same level of EE. Thus, these data reveal an uncoupling between physical activity and global EE for FRW mice, suggesting different specific metabolic adaptations related to activity. The analysis of RER and FA oxidation was done to explore this further.

On D15, the data showed that RER was not affected by food restriction or activity in any group during the overall dark period (Fig. 8A). However, RER determined each hour showed that food delivery was followed by a significant increase in RER in FR and FRW mice between 2100 and 0100 compared with AL and ALW mice (food effect, P < 0.05; Fig. 8B), with values reaching 1.0, which is generally taken to represent both carbohydrate oxidation and endogenous fatty acid synthesis. Intriguingly, from 0700 to 1900, FR mice showed a progressive and significant decrease in RER values that reached ~0.7, suggesting a shift to lipid oxidation compared with FRW mice (P < 0.05; Fig. 8B) that maintained a RER pattern similar to that of the other groups. During the day, an effect of activity was noted (F1,36 = 12.99, P < 0.001; Fig. 8A). Such data were corroborated by the evaluation of fat oxidation (Fig. 8, C and D). During the night, an interaction of food × activity (F1,36 = 5.64, P < 0.05; Fig. 8C) revealed that FRW mice had a lower fat oxidation than ALW mice (P < 0.001) as well as FR compared with AL mice (tendency P = 0.07). However, the high locomotor activity of ALW compared with AL mice during the night is associated with a similar RER, but the FA oxidation values revealed a higher consumption of both lipid and carbohydrates. FRW and FR mice exhibited the same RER.
Fig. 7. Time course of effect of the short- and long-term protocols on energy expenditure. Mean energy expenditure was calculated from dark (1930 to 0730) and light (0730 to 1930) periods on D15 (A and B) and D45 (C and D). Values are expressed as means ± SE. AL, n = 8; ALW, n = 10; FR, n = 10; FRW, n = 12 for the short-term and n = 10 for the long-term protocols. *P < 0.05, FR or ALW vs. AL; #P < 0.05, FRW vs. ALW; §P < 0.05, FR vs. FRW. Significance was set following 2-way ANOVA.

As ALW and AL mice, but their low FA oxidation values suggested a lowered use of lipid and carbohydrate stores. During the light period, FR and FRW mice displayed a lower fat oxidation than their control AL and ALW mice (food effect, F1–36 = 17.63, P < 0.001; Fig. 8C). These data suggest that food restriction increases the total energy derived directly from FA oxidation, especially for FR mice compared with AL, ALW, and FRW mice. On D45, the main changes concerned RER values obtained during the light period, where a food effect was prominent (F1–26 = 25.74, P < 0.001; Fig. 8E and F). Throughout the light phase, FRW mice exhibited the lowest fat oxidation levels compared with all other groups (tendency to interaction of food × activity, F1–26 = 3.71, P = 0.06; Fig. 8, G and H). In restricted mice, physical activity balanced the use of carbohydrate and lipids in the short term, whereas when the protocol was extended it did not impact significantly on lipid or carbohydrate metabolism.

To explore the mechanisms further we measured different plasma metabolites, which can reflect energy balance. Glycemia was performed in the basal condition in the morning (mice were all given the same amount of food 14 h before the measurements) or after 14 h of fasting. On D15, both FR and FRW groups displayed a decreased glycemia in basal or fasted conditions (food effect, F1–67 = 73.39, P < 0.001, and F1–36 = 14.82, P < 0.001, respectively; Table 2). On D55, food and activity affected the basal glycemia (food effect: F1–43 = 45.53, P < 0.005; activity effect: F1–43 = 8.58, P < 0.001; Table 2). Regarding the glycojen stock in liver, food restriction induced a significant decrease only on D15 (food effect, F1–23 = 4.81, P < 0.05; Table 2). Likewise, only at D55 were the plasma levels of ketone bodies affected by both the food restriction and activity (food effect: F1–25 = 13.33, P < 0.001; activity effect: F1–25 = 5.63, P < 0.05; Table 2). The NEFA plasma levels were increased both in FR and FRW mice in the short term (food effect: F1–23 = 8.35, P < 0.01) and the long term (food effect: F1–25 = 6.39, P < 0.05). Finally, no significant difference was detected in the short- and long-term protocols for the plasma triglycerides (Table 2).

Overall, these results show that FR and FRW groups adopt differential energy metabolism strategies. Interestingly, in FRW mice, activity is not related to a higher EE compared with ALW. Moreover, on D15, contrary to FR mice, FRW mice exhibit a similar metabolism to AL and ALW groups during the light phase. Finally, in the long term, FRW mice displayed a similar metabolic profile (RER, FA oxidation) to that obtained in FR mice.

Physical activity and food restriction increased glucose tolerance. Similar to the other energy metabolism parameters, data on glucose tolerance in AN patients during the course of their disease are sparse (57). On D15, FR and FRW mice exhibited a lower glycemia compared with AL and ALW (at time 0, food effect: F1–36 = 14.82, P < 0.001; AUC, food effect: F1–36 = 43.97, P < 0.001). Interestingly, at time 30 min, FRW mice displayed a significantly lower glycemia after glucose injection compared with FR mice (interaction of food × activity: F1–36 = 3.94, P < 0.05; Fig. 9A). On D50, FR and FRW mice exhibited a lower glycemia than AL and ALW (at time 0, food effect: F1–36 = 14.82, P = 0.08; AUC, food effect: F1–36 = 37.28, P < 0.001). No difference remained between FR and FRW groups (Fig. 9B).

Here again, physical activity appears to have beneficial effects by enabling a better adaptation to food restriction with a more rapid and more efficient use of the glucose immediately available, but only in the short term.
Fig. 8. Time course of effect of protocol on respiratory exchange ratio and fat oxidation measured in metabolic cages (n = 1/cage) at different times of the experiment (D15 and D45). Respiratory exchange ratio at short term (A and B) and long term (E and F) was calculated from 4 measurements/h and averaged from CO2 and O2 ratio. Fat oxidation in the short term (C and D) and long term (G and H) was calculated as indicated in METHODS. Mean dark and light RER and fat oxidation were calculated from values obtained between 1930 and 0730 and 0730 and 1930, respectively. Values are expressed as means ± SE. AL, n = 8; ALW, n = 10; FR, n = 10; FRW, n = 12 for the short-term and n = 10 for the long-term protocols. *P < 0.05, FR or ALW vs. AL; #P < 0.05, FRW vs. ALW. Significance was set following 2-way ANOVA.
Physical activity and food restriction modified energy metabolism hormone levels. In AN, patients also display numerous endocrine alterations that can contribute directly or indirectly to maintain and exacerbate the disease (24, 66). Data related to the understanding of how hormones might contribute to the worsening of the patients are currently lacking. On D15 and D55, the plasma levels of leptin were impacted by both activity and food interaction of food × activity, F1–18 = 4.46, P < 0.05, and interaction of food × activity, F1–31 = 12.92, P < 0.001, respectively; Table 3), with significant differences noted between AL and ALW mice (P < 0.05) and AL compared with FR mice (P < 0.05). The plasma levels of corticosterone were increased in FR and FRW mice compared with their respective controls on D15 (food effect, F1–28 = 16.15, P < 0.001; Table 3). On D55, FR mice exhibited the higher corticosteronemia compared with AL and FRW mice (interaction of food × activity, F1–20 = 11.06, P < 0.01; Table 3). Plasma levels of ghrelin, the only known circulating hormone that increases food intake, were increased in the FR and FRW groups compared with AL and ALW mice on D15 (food effect, F1–26 = 37.72, P < 0.001; Table 3) and on D55 (food effect, F1–23 = 4.76, P < 0.05; Table 3). More precisely, on D15, the acylghrelin form was influenced by the food restriction and activity (food effect, F1–26 = 21.48, P < 0.001; activity effect, F1–26 = 6.24, P < 0.05), whereas des-acylghrelin concentrations were increased in FR and FRW mice compared with AL and ALW mice (food effect, F1–26 = 36.81, P < 0.001; Table 3). Thus, the acylghrelin/des-acylghrelin ratio reflected more an impact of activity (activity effect, F1–26 = 10.42, P < 0.01; Table 3) than of the food restriction. On D55, acyl- and des-acylghrelin plasma levels were influenced by activity (activity effect, F1–23 = 8.01, P < 0.01) and food, (food effect, F1–23 = 8.32, P < 0.01; Table 3), respectively, with a higher acylghrelin/des-acylghrelin ratio in ALW and FRW mice (food effect, F1–23 = 5.45, P < 0.05; activity effect, F1–23 = 14.61, P < 0.001; Table 3).

Therefore, our data underscore an impact of physical activity on the ghrelin system. The food restriction led to endocrine changes that could explain the differential adaptations described previously in FR and FRW mice compared with the ad libitum groups.

### DISCUSSION

This study was undertaken to obtain, in a mouse model, data contributing to determine whether the physical activity often described in restrictive AN patients might have beneficial or deleterious effects on bodily functions in the long term. For this purpose, we describe for the first time the physiological consequences of the combination of long-term food restriction with voluntary physical activity in female mice. The main finding is that physical activity induced differential metabolic and endocrine adaptations in chronic food-restricted animals.

Considering the different known animal models related to food restriction and activity and their limitations, we modified the initial ABA model to study lasting alterations. We chose to reduce the quantity of food from 30% for the first days to 50% until the end of the protocol, as normal mice are usually 30% overfed, taking into account their physiological needs (4).

We showed rapid body weight stabilization in the 30% restricted and active mice. Following this 30% restriction, the higher diet restriction combined with physical activity induced a more drastic and a more rapid body weight loss in the short term (up to D15). Then, FRW mice showed 2 wk of stabilized body weight around 20% under their initial body weight. This decrease matched that described previously in long-term 50% FR mice (27). After these 2 wk of stabilization, a slight body weight gain was observed (from D32 to D35 and D40). Then, body weight remained unchanged until the end of the protocol (D55). The body composition revealed that fat mass was strikingly decreased in FRW mice, whereas FR and FRW mice showed a moderate reduction in the lean mass and completely stopped bone mass acquisition.

To search for the causes of this pattern of body weight changes observed only in FRW mice, we studied the physical activity of mice throughout the protocol. First, FRW mice showed a daily activity equivalent to that of ALW mice, suggesting that our model did not induce a hyperactive behavior but rather a moderate activity. Second, the period of slight body weight gain in FRW mice took place when physical activity became lower compared with the beginning of the protocol. This could be related to the weight increase. Further analysis of activity patterns revealed that before their activity became lower, FRW mice were active during both the night.

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**Table 2. Impact of food restriction associated or not with physical activity on plasma metabolites and hepatic glycogen**

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>AL (n = 8)</th>
<th>ALW (n = 6)</th>
<th>FR (n = 8)</th>
<th>FRW (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short term (D15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal glycemia, mg/dl</td>
<td>137.59 ± 7.83</td>
<td>129.39 ± 5.52</td>
<td>86.67 ± 3.63</td>
<td>84.61 ± 4.79</td>
</tr>
<tr>
<td>Fasted glycemia, mg/dl</td>
<td>130.63 ± 8.11</td>
<td>120.80 ± 6.84</td>
<td>107.85 ± 3.60</td>
<td>99.92 ± 4.16</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>33.75 ± 2.85</td>
<td>24.78 ± 2.49</td>
<td>31.5 ± 3.71</td>
<td>28.40 ± 4.00</td>
</tr>
<tr>
<td>NEFA, mg/dl</td>
<td>8.98 ± 0.71</td>
<td>11.46 ± 1.63</td>
<td>14.49 ± 1.52</td>
<td>13.69 ± 1.37</td>
</tr>
<tr>
<td>Ketone bodies, mg/dl</td>
<td>4.01 ± 0.69</td>
<td>5.14 ± 0.84</td>
<td>3.18 ± 0.57</td>
<td>4.04 ± 0.56</td>
</tr>
<tr>
<td>Liver glycogen, mg/mg tissue</td>
<td>9.14 ± 0.86</td>
<td>9.33 ± 0.95</td>
<td>7.37 ± 0.47</td>
<td>7.56 ± 0.74</td>
</tr>
<tr>
<td>Long term (D55)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal glycemia, mg/dl</td>
<td>119.93 ± 5.15</td>
<td>131.17 ± 5.70</td>
<td>82.85 ± 4.08</td>
<td>100.86 ± 3.86</td>
</tr>
<tr>
<td>Fasted glycemia, mg/dl</td>
<td>119.56 ± 7.73</td>
<td>105.80 ± 4.96</td>
<td>99.15 ± 6.28</td>
<td>106.88 ± 3.00</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>33.03 ± 2.52</td>
<td>26.40 ± 1.98</td>
<td>26.02 ± 2.53</td>
<td>27.86 ± 3.28</td>
</tr>
<tr>
<td>NEFA, mg/dl</td>
<td>12.94 ± 1.40</td>
<td>17.34 ± 0.98</td>
<td>21.20 ± 2.58</td>
<td>18.85 ± 2.16</td>
</tr>
<tr>
<td>Ketone bodies, mg/dl</td>
<td>4.11 ± 0.33</td>
<td>5.53 ± 0.72</td>
<td>3.00 ± 0.25</td>
<td>3.58 ± 0.36</td>
</tr>
<tr>
<td>Liver glycogen, mg/mg tissue</td>
<td>10.24 ± 1.56</td>
<td>8.91 ± 0.96</td>
<td>10.18 ± 0.52</td>
<td>10.82 ± 0.59</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = 6–8/group. NEFA, nonesterified (or free) fatty acids All samplings were done in the morning (830–1100). Significance was set following 2-way ANOVA. *Food effect; †activity effect.
and the day throughout the protocol, whereas activity in all other groups was restricted to the dark period. FRW mice developed a light period activity close to the feeding time. This activity, usually called FAA, was also described in the ABA paradigm (23, 48, 49, 68). The FAA is usually described 2–3 h before food distribution. In our model, FRW mice began to be active around 6 h before food on D15. Such behavior might reflect an association between foraging and FAA. We hypothesized that the peak of activity observed 2–3 h before feeding might really correspond to FAA, whereas the previous activity might be related to foraging. Tentatively, we suggest that wheel running impacts on the development of FAA by increasing addictive behavior to exercise (37). Indeed, FAA is driven by the dopaminergic motivation/reward system (31, 67). Alterations of the reward system are increasingly suspected to play a role in keeping AN patients in a vicious circle of food restriction associated usually with activity (5, 60). Finally, until the end of the protocol, FRW mice maintained a moderate activity, even if a strong decrease that resulted in a reduction in their global daily activity was observed for the FAA.

The relation between leptin and physical activity has been well investigated, especially in the ABA protocol (25, 33, 51). In fasted mice, increases in ambulatory and running wheel activity have been shown to be inhibited by chronic subcutaneous leptin treatment (51). In the ABA protocol, subcutaneous or intracerebroventricular leptin delivery leads to a decrease in running wheel activity (25, 33). In our study, on D15 as well as on D55, FRW, ALW, and FR mice had a similar low plasma leptin levels compared with AL mice, reflecting their fat mass reduction, especially the visceral fat pads. In accordance with the known effect of leptin on activity, the slight increase in plasma leptin levels described in FRW mice between D15 and D55 might be a contributing factor to the disappearance of the FAA at D55. Of note is that AN patients also exhibit lower plasma levels of leptin in association with their fat mass reduction (50). Our data reinforce the importance of studies exploring the impact of food restriction in association with activity in chronic long-term protocols.

Ghrelin is another hormone involved in FAA behavior (68). As for AN restrictive-type patients (29, 30), on D15 the two groups of food-restricted mice displayed an increase in plasma acyl and des-acylgrehelin levels. The ratio acyl/des-acylgrehelin contributes to a better idea of the potential ghrelin activity because of the controversial functions of these two forms that have been discussed (17). In our study, on D15, ALW and FRW mice displayed a higher acyl/des-acylgrehelin ratio compared with AL and FR mice, suggesting that active ghrelin

![Fig. 9. Impact of food restriction associated or not with physical activity on intraperitoneal glucose tolerance. Blood glucose variations were measured following 1.5 g/kg bolus glucose injection in the short term (A) and long term (B) after 12–14 h of fasting, at time (t)15, 30, 60, 90, and 120 min. Values are expressed as means ± SE. AL, n = 8; ALW, n = 10; FR, n = 10; FRW, n = 10. *P < 0.05, FR or ALW vs. AL; #P < 0.05, FRW vs. ALW; §P < 0.05, FR vs. FRW. Significance was set following 2-way ANOVA. IPGTT, intraperitoneal glucose tolerance test.](image-url)

### Table 3. Impact of food restriction associated or not with physical activity on plasma hormones

<table>
<thead>
<tr>
<th>Hormones</th>
<th>AL (n = 6–8)</th>
<th>ALW (n = 6–8)</th>
<th>FR (n = 6–8)</th>
<th>FRW (n = 6–8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Short term (D15)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>5.72 ± 0.87</td>
<td>3.09 ± 0.21*</td>
<td>3.55 ± 0.25*</td>
<td>3.08 ± 0.20</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>147.1 ± 20.10</td>
<td>196.4 ± 24.45</td>
<td>271.9 ± 42.59</td>
<td>276.4 ± 43.01</td>
</tr>
<tr>
<td>Ghrelin total, pg/ml</td>
<td>907.34 ± 111.75</td>
<td>969.4 ± 96.24</td>
<td>2,158.2 ± 177.54</td>
<td>2,721.3 ± 396.71</td>
</tr>
<tr>
<td>Acylghrelin, pg/ml&lt;sup&gt;b&lt;/sup&gt;</td>
<td>228.70 ± 35.38</td>
<td>357.09 ± 60.68</td>
<td>575.60 ± 63.66</td>
<td>958.21 ± 165.69</td>
</tr>
<tr>
<td>Des-acylgrehelin, pg/ml</td>
<td>678.64 ± 79.80</td>
<td>612.34 ± 72.49</td>
<td>1,582.56 ± 177.54</td>
<td>1,763.14 ± 252.69</td>
</tr>
<tr>
<td>Ghrelin ratio (AG/DAG)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.34 ± 0.03</td>
<td>0.62 ± 0.09</td>
<td>0.39 ± 0.06</td>
<td>0.54 ± 0.06</td>
</tr>
<tr>
<td><strong>Long term (D55)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>4.67 ± 0.42</td>
<td>3.17 ± 0.15*</td>
<td>3.37 ± 0.16*</td>
<td>3.52 ± 0.09</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>100.8 ± 11.60</td>
<td>120.5 ± 10.19</td>
<td>240.8 ± 21.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>178.0 ± 12.766</td>
</tr>
<tr>
<td>Ghrelin total, pg/ml</td>
<td>1,238.6 ± 151.1</td>
<td>1,458.1 ± 177.8</td>
<td>1,605.9 ± 112.5</td>
<td>1,774.4 ± 165.46</td>
</tr>
<tr>
<td>Acylghrelin, pg/ml&lt;sup&gt;b&lt;/sup&gt;</td>
<td>145.48 ± 42.12</td>
<td>277.44 ± 44.02</td>
<td>147.21 ± 20.26</td>
<td>240.34 ± 41.46</td>
</tr>
<tr>
<td>Des-acylgrehelin, pg/ml</td>
<td>1,093.12 ± 114.96</td>
<td>1,180.63 ± 135.48</td>
<td>1,458.69 ± 108.39</td>
<td>1,534.10 ± 132.16</td>
</tr>
<tr>
<td>Ghrelin Ratio (AG/DAG)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.12 ± 0.03</td>
<td>0.23 ± 0.01</td>
<td>0.10 ± 0.02</td>
<td>0.15 ± 0.02</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = 6–8/group. AG, acylghrelin; DAG, des-acylgrehelin. All samplings were done in the morning (830–1100). *P < 0.05, FR or ALW vs. AL; §P < 0.05, FR vs. FRW. Significance was set following 2-way ANOVA. <sup>a</sup>Food effect; <sup>b</sup>activity effect.

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levels might impact upon physical activity or vice versa (48). In the long term, this ratio in FRW mice decreased and became similar to that of FR and AL mice. This decrease was associated with lowered FAA in FRW mice. Interestingly, in the ABA protocol, the stimulatory action of acyl-ghrelin exerted on FAA is abolished by chronic intracerebroventricular growth hormone secretagogue receptor type 1a antagonist treatment (68).

Finally, corticosterone plasma levels are increased in AN patients (29). Besides its known role in the regulation of stress response, corticosterone was demonstrated to contribute to the development of FAA in rodents submitted to the ABA protocol (22). In the short-term protocol, FRW and FR exhibited increases in plasma levels of corticosterone. However, in the long-term protocol, FR mice displayed higher levels than AL and FRW mice. Such changes might explain the slight activity observed just around the food distribution for FR mice and again might be one possible explanation for the disappearance of FAA in FRW mice. Once again, such differences between the two restricted groups might explain the differential pattern of activity. Thus, the data we have obtained gave us two main novel findings. 1) FRW mice showed a unique pattern of leptin, ghrelin ratio, and corticosterone that is involved in the regulation of physical activity but also in the regulation of energy metabolism; and 2) voluntary and moderate physical activity at the beginning of a protocol of food restriction might be beneficial in the long term since it is associated with a normalization of metabolic hormone levels that may favor a better adaptation to these drastic conditions.

The low energy expenditure observed in FR and FRW mice on D15 and D45 might be associated with low plasma leptin concentrations but also with the food restriction since ALW mice, which also showed low levels of leptin, exhibited high EE during the night. Accordingly, the reduction of lean mass observed in FR and FRW mice might be a strategy to spare energy used by the organism in such drastic conditions to avoid a too dramatic hypothermia (62). It might be associated with the persistent high corticosterone levels, which are known to simulate conversion of protein to glucose and increase nitrogen catabolism in muscle. It can also be hypothesized that fat pad decreases might impact EE through the decrease of plasma leptin levels. Indeed, leptin treatment in fasted male mice increased total EE (51). The impact of ghrelin on EE was not clear since intracerebroventricular or intraperitoneal injection of ghrelin decreased EE (3), whereas intraperitoneal treatment with proghrelin increased EE (74). Such data point out the controversial dual effect of acylghrelin and des-acylghrelin on food intake and EE. Here, our data did not permit us to decipher the real impact of ghrelin on EE. Finally, the low EE described in both FR and FRW mice, especially during the light period when only FRW showed FAA, led us to conclude that EE not only was related to exercise but reflected an adaptive way to save energy to maintain physiological functions. The impact of food restriction on energy metabolism confirmed data obtained with 30% of food restriction (8). However, the strategy used by FR and FRW mice were different. In fact, in the short term, FRW mice adopted different fuel utilization from FR during the light period. FR mice displayed a metabolism that was oriented to FA oxidation, which was not the case for FRW mice despite the food restriction and the FAA.

These metabolic data suggest that, in the short term, FRW mice did not adapt properly to their metabolism, with a higher carbohydrate metabolism and a lower fat oxidation during the light period than FR mice. Such a profile was supported by the results obtained in the glucose tolerance test; FRW mice displayed a faster glucose clearance particularly just after glucose injection. This improved glucose capture could explain why FRW mice have a higher RER than FR during the day on D15. When the protocol was extended, FRW mice adopted a similar metabolic profile to FR mice. Thus, physical activity may delay the metabolic adaptations to food restriction even if the other parameters (such as liver weight, hepatic glycogen, basal and fasted glycemia, NEFA, leptin, corticosterone, and ghrelin) evolved in a similar way in FR and FRW in the short term. In the long term, FRW mice appeared to be more adapted to these drastic conditions compared with FR mice. Besides the increased body weight, FRW mice had lower corticosterone and acylghrelin levels compared with D15, whereas they remained constant in the FR group. Because of their role in the regulation of energy metabolism, glycemia, and food intake, further studies are needed to explore the precise role of these two key hormones in the adaptation process of FRW mice. To our knowledge, very few studies have focused on metabolite concentrations related to energy metabolism in AN (13, 55, 72), likely reflecting the discrepant data obtained and the procedures used to measure these metabolites (times of the day, before or after a meal, fasted conditions, etc.), rendering the data difficult to interpret. This underscores the necessity to study the evolution of these parameters in relevant animal models.

It is known that physical activity is generally associated with fuel mobilization, reflected by increases in glycemia and plasma NEFA, as well as feeding that participated in an overload of blood nutriments (59). We showed here that running wheel activity modified the pattern of ingested food in FRW mice in shifting the meal initiation. FRW mice ate the delivered food more slowly than FR mice. One explanation, suggested by Woods (71), considered eating to be a homeostatic stressful event, because the digested nutriments that reached the blood during and after a meal markedly disrupt energy homeostasis. Thus, the combination of both events, activity and feeding, could generate a stressful energy event especially in the short term, where FRW mice delayed their time to eat. Thus, the shift in meal initiation observed here might explain the “voluntary food restriction” observed in the ABA protocol (38). In fact, no study has measured the meal initiation in the ABA model, in which food is available for only 2 h. Taken together with our data, we hypothesize that the voluntary food restriction described in the ABA model might be due to the physical activity maintained by the mice that could modify the beginning of feeding. Another critical factor in the ABA model is the dehydration. In contrast to what was observed in the ABA protocol, in which the consumption of water could play a role in the reduction of food intake (7), we did not observe any modification in the water intake pattern. Thus, here physical activity influenced the meal initiation without affecting the water consumption. It can be hypothesized that the moderate and not the high level of activity described in our model is responsible for this observation.
Food restriction is also known to disturb reproduction function both in humans and rodents. As demonstrated by Dos Santos et al. (21), female rats submitted to treadmill exercise and a 50% food restriction led to interruption of estrous cyclicity, as we observed in our study where the physical activity was voluntary. In AN patients, even if amenorrhea was not considered as a criterion in the DSM-5 anymore, disruption of cycles is currently observed in restrictive AN patients (24, 42). Disruption of the estrous cycle as well as reduction of the size of ovaries was observed here in FR and FRW mice along all the protocols. Physical activity alone did not promote such alteration. The combination of energy deficit and activity seems necessary to induce these modifications. These alterations might also be adaptive to avoid the development of offspring in a deleterious nutritional environment. The endocrine changes observed in our study might contribute to the development of this phenotype. Leptin was largely described to play an essential role in reproduction since db/db mice, which are deleted for leptin gene receptor, and ob/ob mice, which are deleted for leptin gene, are infertile (6, 18). Moreover, upregulation of ghrelin induced a decrease in LH surge (26, 53), even if the mechanisms involved are yet unknown. Chronic exposition to corticosterone inhibited LH secretion and estrous cycle and ovulation in female rodents (9, 10). Thus, both FR and FRW mice displayed high plasma corticosterone and ghrelin levels as well as a strong fall in the leptin concentrations. Therefore, the various endocrine alterations observed in our study are likely to alert hypothalamic brain structures involved in reproductive function and energy metabolism of the energy imbalance to preserve sufficient energy to adapt the body both at short- and long-term protocols. Finally, the body weight gain observed in FRW mice after 30 days of protocol did not induce a recovery of the estrous cycle. Indeed, it remained absent until the end of the long-term protocol.

Finally, another major alteration of AN is a decrease in BMC leading to osteopenia and osteoporosis in 38–50% of cases, which is associated with the high risk of fracture in patients (41–43). Alterations in BMC were described in rats and mice aged 3–14 wk and subjected to protocols where food restriction varied from 30 to 40% for 3–10 wk (19, 32, 54, 63). In FR and FRW mice, bone mass acquisition was stopped similarly after D15, whereas it was increased for AL and ALW mice. Thus, in FRW mice, physical activity that is usually described to stimulate bone formation did not prevent the termination of bone mass acquisition induced by food restriction. Similar data were also described in female mice subjected to a protocol of chronic stress associated with caloric restriction, the “separation based-anorexia” model (73). This confirmed the absence of the protective effect of activity on BMC in AN.

In conclusion, here we have characterized a new rodent model of chronic restriction associated with voluntary physical activity that mimics, at least in part, the physiological alterations that occur in AN. This model was used to determine whether physical activity has a beneficial or detrimental role in this disease in the long term. Our data lead us to suggest a dual effect of exercise. Moderate voluntary exercise accelerates body weight loss (particularly affecting the fat mass), leading to noticeable endocrine alterations, which could impact food anticipatory activity, effects also observed in AN patients. It did not permit a proper metabolic adaption in the short term with a metabolism similar to that of ad libitum groups. However, a delayed food intake initiation due to physical activity limited a rapid food intake pattern, which was observed only in FR mice. This behavior resembles the binge eating behavior observed in some AN patients, which causes abdominal discomfort and possibly also lower nutrient adsorption. In this case, physical activity might have a beneficial effect. In the long term, we suggest that moderate exercise may have positive effects on energy metabolism regulation. Finally, the evolution of hormone levels noted in both the short term and long term reflected an effect of both activity and food restriction on adaptation to these drastic conditions of negative energy balance, with a potential positive effect in the long term. However, activity did not contribute to prevent lean and bone mass loss induced by food restriction. It remains to be determined whether these specific physiological adaptations in our AN model (metabolic, endocrine) that are related to moderate activity could lead to a better or poorer recovery.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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