Nature and nurture effects of voluntary activity and nutrition on energy balance and nutrition

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EFFECTS OF SELECTIVE BREEDING FOR INCREASED WHEEL RUNNING BEHAVIOR ON CIRCADIAN TIMING OF SUBSTRATE OXIDATION AND INGESTIVE BEHAVIOR

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Summary

Fluctuations in substrate preference and utilization across the circadian cycle may be influenced by the degree of physical activity and nutritional status. In the present study, we assessed these relationships in control mice and in mice from a line selectively bred for high voluntary wheel-running behavior, either when feeding a carbohydrate-rich/low-fat (LF) or a high-fat (HF) diet. Housed without wheels, selected mice, and in particular the females, exhibited higher cage activity than their non-selected controls during the dark phase and at the onset of the light phase, irrespective of diet. This was associated with increases in energy expenditure in both sexes of the selection line. In selected males, carbohydrate oxidation appeared to be increased compared to controls. In contrast, selected females had profound increases in fat oxidation above the levels in control females to cover the increased energy expenditure during the dark phase. This is remarkable in light of the finding that the selected mice, and in particular the females showed higher preference for the LF diet relative to controls. It is likely that hormonal and/or metabolic signals increase carbohydrate preference in the selected females, which may serve optimal maintenance of cellular metabolism in the presence of augmented fat oxidation.

1. Introduction

Food intake of animals is regulated to match the energetic demands on the long-term, but the regulatory mechanisms exert their influences on a daily basis, and even within each meal (Li & Anderson 1982;Shor-Posner, Brennan *et al.* 1994). Since life evolved on a planet with an approximately 24h rotation, most animal species have a circadian rhythm in activity and resting, with specific metabolic and nutritional demands integral to the regulation of energy balance. As such, most species, including human beings and rodents, eat the majority of their food during the active phase, with peaks occurring at the beginning and at the end of the activity phase (Strubbe & van Dijk 2002). Besides total amount of food eaten, macronutrient preference also fluctuates across the daily cycle. The beginning of the circadian phase is usually associated with an increased appetite for carbohydrates, whereas at the end of the active phase particular fats are preferred (Shor-Posner, Brennan *et al.* 1994;Tempel, Shor-Posner *et al.* 1989).

It may be proposed that these respective macronutrient preferences exist to serve metabolic needs across the diurnal cycle. At the onset of the active phase, for example, carbohydrates may be needed because they can rapidly deliver energy for behavioral arousal (Strubbe & van Dijk 2002). Fats, on the other hand, are metabolized slowly and would not be a preferred fuel at the onset of the active phase. Instead, they could be more appropriate at the end of the active phase, as they can be stored more easily and subsequently yield fuels during the inactive phase (Strubbe & van Dijk 2002). In the present study, we investigated relations between circadian timing of ingestive behavior, metabolism (relative oxidation of carbohydrates and fats), and macronutrient preference at the beginning and at the end of the active phase. This was done in mice selectively bred for high voluntary wheel-running behavior, and in their non-selected controls. In previous studies, we noticed that these selectively bred, highly active mice have increased energy intake with a relatively low body weight compared to controls (Vaanholt, Jonas *et al.* 2008;Swallow, Koteja *et al.* 2001). The high energy intake is probably necessary to match the increased energy utilization associated with increased physical activity. Furthermore, when physical activity-selected mice were switched to feeding a high-fat diet, they - particularly the females - were obesity-resistant despite the fact that the selected female mice further increased their food intake relative to when they were feeding a carbohydrate-rich, low-fat diet (Vaanholt, Jonas *et al.* 2008). It may be suggested that it is either a response to the high energy demand of the high spontaneous activity or they simply prefer the high-fat diet. Because the activity-selected mice have an increased level of spontaneous activity even without the presence of running wheels, particularly in their active phase (Rhodes, Hosack *et al.* 2001;Malisch, Breuner *et al.* 2009), we hypothesized that these selected mice would have increased food intake at the onset of the dark phase, with a stronger preference for carbohydrates than control mice to serve their metabolic requirements. We predicted that these preferences would be augmented when animals were switched to feeding a high-fat diet.

2. Materials and methods

2.1. Animals

Mice used in this study were selected for voluntary wheel-running behavior (the base population was the Hsd:ICR strain) over 31 generations and were obtained from T. Garland Jr, Riverside, CA. Breeding lines were maintained at our facilities in Haren without further selection for wheel-running activity. In the original selection protocol, eight lines of mice were created (4 selected and 4 control) (Swallow, Carter *et al.* 1998), and here we used male and female mice at the age of 5 months from one selection line (lab designation line 7) and one control line (line 2). All mice were individually housed in Plexiglas cages (20x20x30) in the same room with *ad libitum* food and water at an ambient temperature of 22±1 °C, and maintained on a 12:12 light-dark cycle with lights on at 8 am. This was designated circadian time (CT) 0, and light off was CT 12. Pine shavings and EnviroDry® were used as bedding material. Half of the animals were either on a pelleted standard low-fat (LF) carbohydrate rich rodent chow (RMH-B 2181, HopeFarms BV, Woerden, NL) or on a pelleted 60 % high fat (HF) diet, which was equicaloric to the standard lab chow (see (Vaanholt, Jonas *et al.* 2008) for diet description). Long-term energy balance parameters in these same mice were published previously (Vaanholt, Jonas *et al.* 2008). All methods were approved by, and are in agreement with the regulations of the Institutional Animal Use and Care Committee of the University of Groningen. These regulations are consistent with the guidelines for the care and use of laboratory animals as described by the U.S. National Institutes of Health.

2.2. Indirect calorimetry

Animals in their homecages were taken to the respirometry room where respirometric chambers were set up to determine oxygen consumption (VO_2, l/h) and carbon dioxide production (VCO_2, l/h). Eight mice could be measured simultaneously. Oxygen and carbon dioxide concentration of dried inlet and outlet air (drier: molecular sieve 3 Å, Merck) from each chamber were measured with a paramagnetic oxygen analyzer (Servomex Xentra 4100) and cardon dioxide by an infrared gas analyzer (Servomex 1440). The system recorded the differentials in oxygen and carbon dioxide between dried reference air and dried air from the metabolic chambers. Flow rate of inlet air (60 l/hour) was measured with a mass-flow controller (Type 5850 Brooks). Ambient temperature in the room and chambers, as well as behavioral activity (with passive infrared detectors, Optex Wonderex FX-35) were measured simultaneously. Samples were collected every 10 minutes (allowing optimal air mixing) for each animal and automatically stored on a computer. To reduce novel cage stress, the respirometric chambers (45x25x30 cm) were adapted to accommodate the home cage of the animal. Animals therefore did not need to be handled and stayed in their home cage during the entire measurements. Animals were measured at an ambient temperature of 22°C and food and water were provided *ad libitum* over a period of 48 hrs. Energy expenditure (kJ/h) was calculated using the following equation of Ferrannini (Ferrannini 1988):

$$EE= \{[(RQ - 0.7) / 0.3] \times 473 + [(1.0 - RQ) / 0.3] \times 439\} \times VO_2$$

where oxidation (by one mol O_2) of carbohydrates and fats yields respectively 473 and 439 kJ. The relative contributions of carbohydrate and fat oxydation to EE were estimated by comparing the assessed Respiratory Quotient (RQ; i.e. VCO_2/VO_2) to the levels when carbohydrate oxidation (RQ = 1.0) or fat oxidation (RQ=0.7) predominate.

Furthermore, the assessed oxygen consumption and carbon dioxide production were used to calculate carbohydrate and fat oxidation according to the equations of Lusk (Lusk G 1976):

Lipid oxidation (g/hr): $38.461 \times \{(VO_2(mol/hr) - VCO_2(mol/hr)\}$

Carbohydrate oxidation (g/hr): $\{94.017 \times VCO_2(mol/hr)\} - \{66.239 \times VO_2(mol/hr)\}$

These formulas are derived from the notion that 0.036 mol and 0.088 mol of O_2 are necessary to oxidize unit masses of carbohydrates and fats, respectively, and 0.036 mol and 0.062 mol of CO_2 are produced upon oxidation of unit masses of carbohydrates and fats, respectively (Lusk G 1976).

2.3. Circadian rhythm of food intake

The circadian rhythm of food intake was obtained by two separate sessions interspaced by 3 days (first session: from CT 0 to CT 12, second session: from CT 12 to CT 0). This was done by manually weighing food hoppers every two hours, and determining the food missing from the hoppers.

2.4. Food preference

All animals that had been adapted to feeding the LF or the HF diet, were given access to an equal amount (approx 10 gr.) of both HF and LF diets for two hours to determine their food preference. This was done once immediately before the dark phase (CT 10 to CT 12), and a week later the measurement was done immediately before the light phase (from CT 22 am to CT 0). These time periods were chosen because at these two distinct phases of the activity period control and selected mice had roughly similar food intakes (see circadian feeding patterns, Fig. 5).

2.5. Statistical analysis

Data were analyzed using GLM repeated measures and between-subjects effects using Statistica 7 program for spontaneous activity, RQ, carbohydrate-, fat oxidation, food intake behavior and energy expenditure. Except for spontaneous activity (to compare gender effects on spontaneous activity), males and females were tested separately due to their difference in energy regulation. Differences in food preferences (indicated by the percentage intake of LF over total intake

assessed over two hours) were tested using GLM Multivariate Analysis. In all tests, a level of $p<0.05$ was considered significant.

3. Results

3.1. Circadian rhythm of spontaneous activity

GLM repeated measures revealed that females were generally more active in the dark phase than males (sex effect, $F_{(1,45)}=7.82$; $p<0.01$) (see Figure 1). Selected mice were more active at the onset of the light phase and throughout the whole dark phase than control mice (line effect, $F_{(1,45)}=17.53$; $p<0.001$), irrespective of sex. These effects were particularly evident in females of the selection line, which were highly active in the dark phase (sex*line effect, $F_{(1,45)}=7.02$; $p<0.05$). No statistically significant effects of diet or a diet*line interaction effects of diet were observed in either sex.
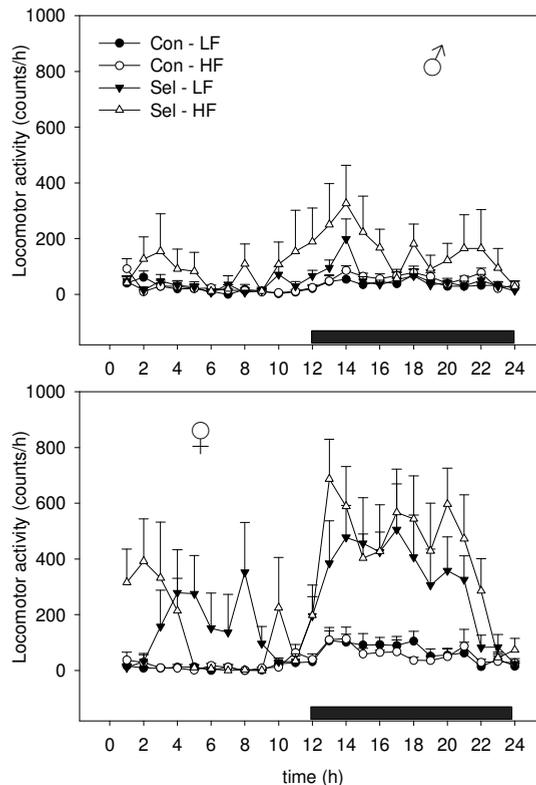

Figure 1. Locomotor activity during indirect calorimetry in control (Con) and selected (Sel) male (upper panel) and female (bottom panel) mice feeding a low-fat (LF) or high-fat (HF) diet. Dark phase is indicated by the black horizontal bar at the bottom of the graph.

3.2. Circadian rhythm of energy expenditure

Over the daily cycle, GLM repeated measures revealed an effect of line in both males $(F(1,18)=7.59; p<0.05)$ and females $(F(1,18)=16.30; p<0.01)$, meaning that selected mice had a higher energy expenditure than control mice irrespective of diet (see Figure 2). Univariate analysis of individual time points revealed that energy expenditure was increased in selected males at CT 3-4, 6-14 and in selected females at CT 4, 7, and 12-20 compared to control animals. No statistically significant effects of diet or a diet*line interaction effects of diet were observed in either sex.
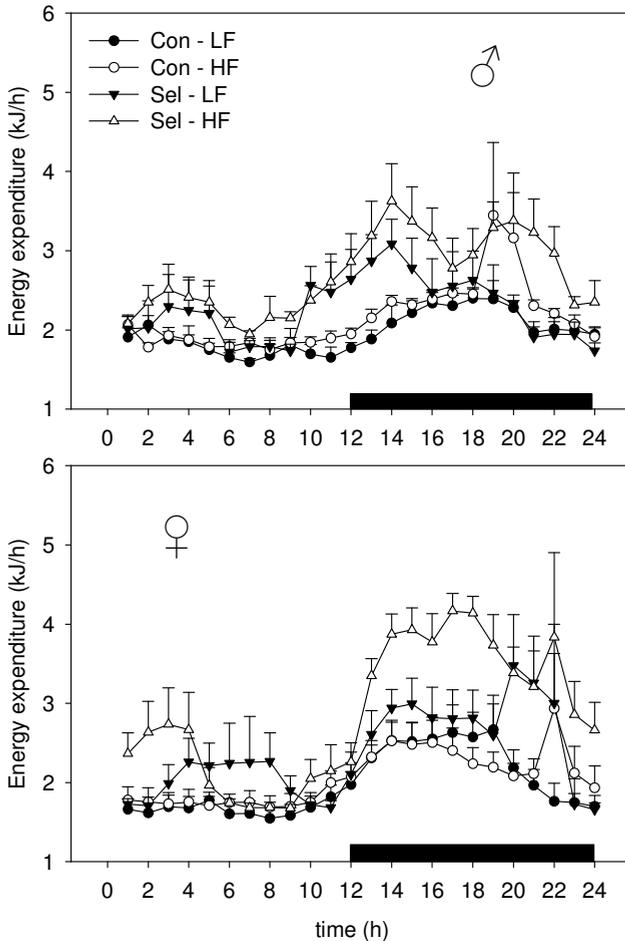

Figure 2. Energy expenditure in control (Con) and selected (Sel) male (upper panel) and female (bottom panel) mice feeding a low-fat (LF) or high-fat (HF) diet. Dark phase is indicated by the black horizontal bar at the bottom of the graph.

3.3. Respiratory Quotient

Over the daily cycle, GLM repeated measures revealed that both male ($F(1,23)=116.76$: $p<0.001$) and female ($F(1,21)=266.08$; $p<0.001$) mice on the HF diet had a lower RQ than mice on the LF diet (see Figure 3). Furthermore, univariate analysis in males pointed out that selected mice had higher RQ values at CT 10 – 14 than controls. Using GLM repeated measures, females of the selection line had decreased RQ levels compared to mice of the control line (line effect, $F(1,21)=11.51$; $p<0.01$) irrespective of diet. This was particularly the case between CT 12 – 15.
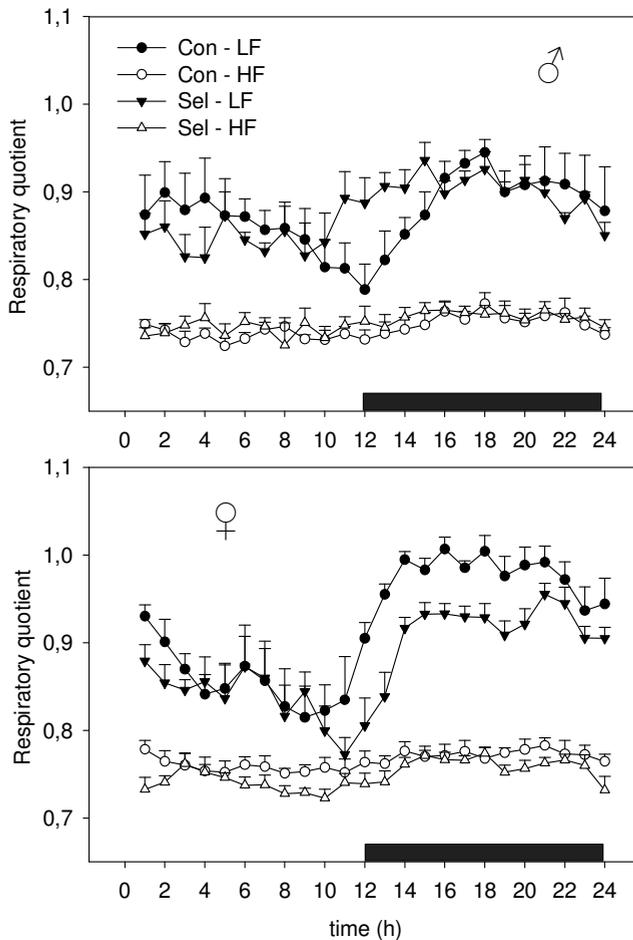

Figure 3. Respiratory Quotient (RQ) values during indirect calorimetry in control (Con) and selected (Sel) male (upper panel) and female (bottom panel) mice feeding a low-fat (LF) or high-fat (HF) diet. Dark phase is indicated by the black horizontal bar at the bottom of the graph.

3.4. Carbohydrate and fat oxidation

Over the daily cyle, GLM repeated measures demonstrated that, compared to the LF condition, feeding the HF diet caused a decreased carbohydrate oxidation in males ($F(1,18)=4.96$; $p<0.05$) and in females ($F(1,18)=95.08$; $p<0.001$) and increased fat oxidation in males ($F(1,18)=7.69$; $p<0.05$) and in females ($F(1,18)=82.22$; $p<0.001$) (see Figure 4). Furthermore, males of the selection line showed increased carbohydrate oxidation ($F(1,18)=6.33$; $p<0.05$) irrespective of diet, and selected females showed an increase in fat oxidation ($F(1,18)=21.04$; $p<0.001$) irrespective of diet compared to mice of the control line. No interaction effects were observed.
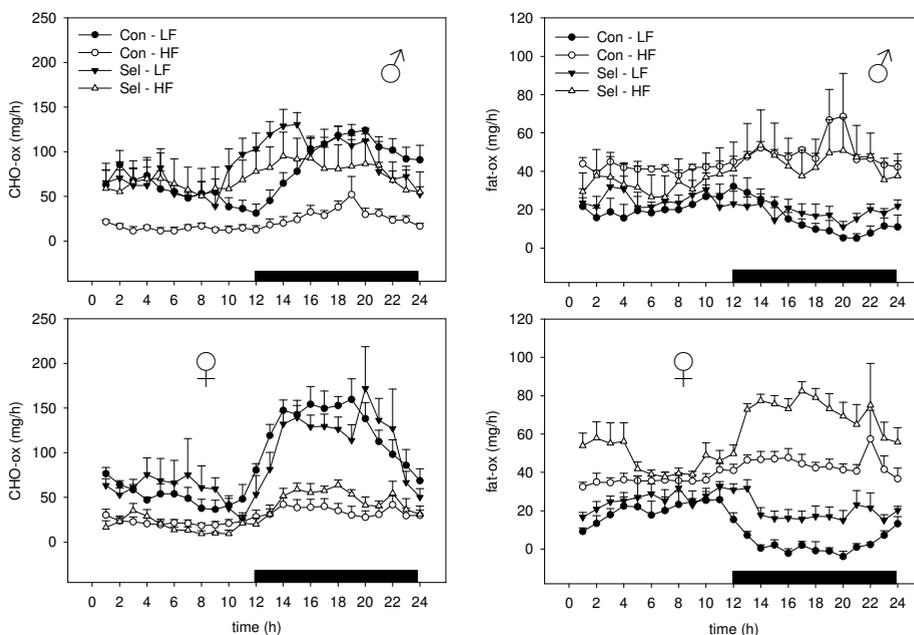
Figure 4. Calculated carbohydrate (CHO; left graphs) and fat (right graphs) oxidation in control (Con) and selected (Sel) male (upper panel) and female (bottom panel) mice feeding a low-fat (LF) or high-fat (HF) diet. Dark phase is indicated by the black horizontal bar at the bottom of the graph.

3.5. Circadian rhythm of food intake

Over the daily cycle, GLM repeated measures revealed in both sexes that selected mice had significantly different intake over time (males: $F(1,24)=14.66$; $p<0.001$; females: $F(1,21)=8.87$; $p<0.01$) relative to control mice (see Figure 5). Univariate analysis showed that selected males

consumed more food than control males at CT 12 and 14, whereas selected females consumed more at CT 6 and 8 than control females, irrespective of diet.
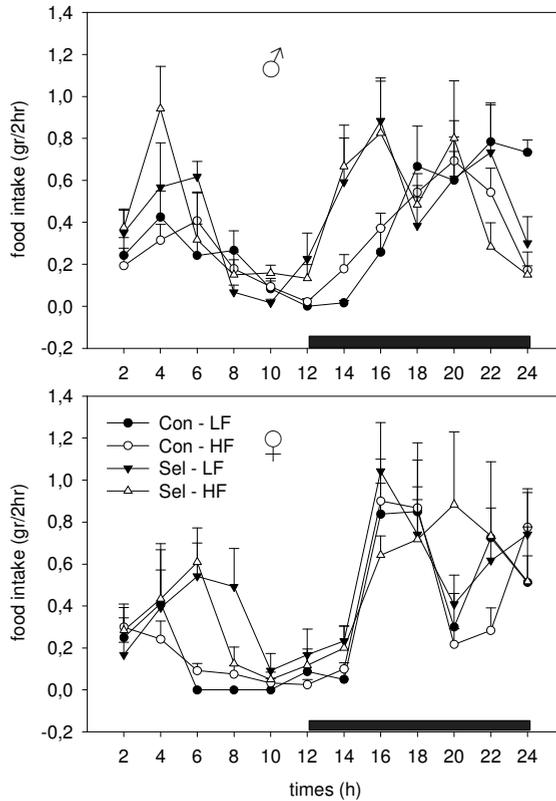

Figure 5. Food intake in control (Con) and selected (Sel) male (upper panel) and female (bottom panel) mice feeding a low-fat (LF) or high-fat (HF) diet. Dark phase is indicated by the black horizontal bar at the bottom of the graph.

3.6. Food selection

In the food selection experiment (calculated as percentual intake of LF diet over intake of LF and HF diet) at two time points of the daily cycle, mice feeding the HF diet ate less than mice feeding the LF diet, both in males ($F_{(1,69)}=14.92$; $p<0.001$) and in females ($F_{(1,45)}=11.32$; $p=0.01$) (see Figure 6). Furthermore, males of the selection line consumed more than those of the control line at the onset of the dark phase (line*time effect: $F_{(1,69)}=4.77$; $p<0.05$), but not at the onset of the light phase. No differences were observed in total intake during the two-choice test between control and selected females.

Mice feeding the HF diet as their "normal" diet generally preferred the LF diet more than mice that had the LF diet as their "normal" diet (males: $F_{(1,69)}=32.97$; $p<0.0001$; females $F_{(1,45)}=15.47$; $p<0.001$). Furthermore, selected females preferred the LF diet more than control females ($F_{(1,45)}=6.76$; $p<0.05$), particularly at the onset of the dark phase, irrespective of diet condition.
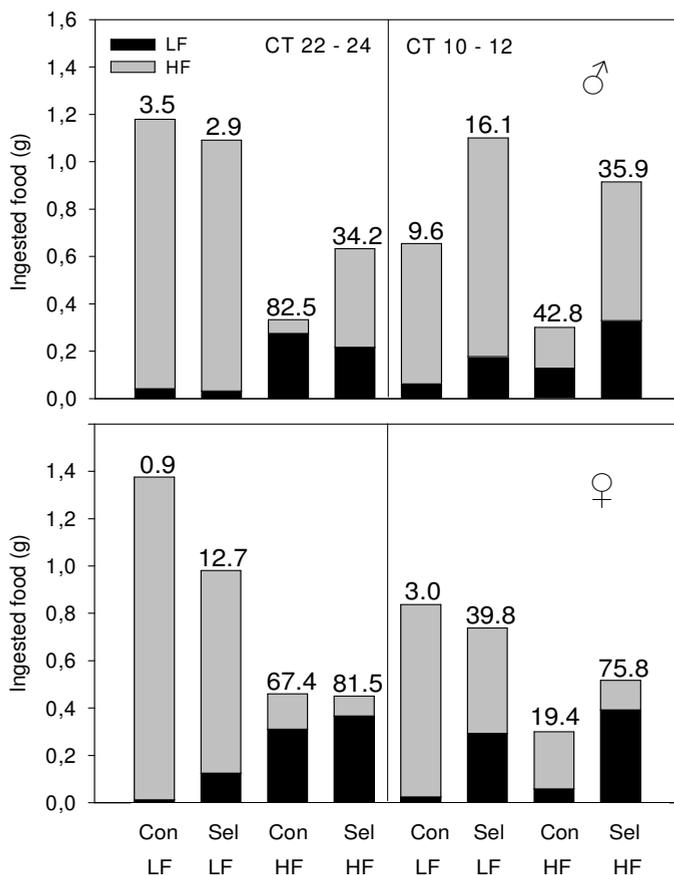
Figure 6. Food selection test in control (Con) and selected (Sel) male (upper panel) and female (bottom panel) mice at CT 22-24 (at the late dark phase, left panels) and at CT 10-12 (at the late light phase, right panels) either habituated to a low-fat (LF) or high-fat (HF) diet. Grey and dark fields of bars indicate respectively HF and LF intake when given two-hour access to these two diets. Numbers above the bars indicate the percentage intake of the LF diet of total.

4. Discussion

The present study was designed to investigate the hypothesis that mice selectively-bred for high voluntary wheel-running behavior exhibit increased food intake at the onset of the dark phase, with a stronger preference for carbohydrates than control mice. We predicted that these behaviors would reflect differences in utilization profiles of carbohydrates and fats.

Relative to controls, the selected mice displayed increased spontaneous activity. This increase was most striking in the dark phase, and the selected females showed a more dramatic increase than the selected males (Fig. 1). Thus, the increased voluntary locomotion by the selected animals is not restricted to wheel-running behavior, and this is consistent with earlier findings (Rhodes, Hosack *et al.* 2001;Malisch, Breuner *et al.* 2009). Furthermore, these data imply that the normal circadian distribution of voluntary activity, with low-level activity in the light phase and high-level activity in the dark phase, is maintained in the wheel-running selected mice (see also (Koteja, Swallow *et al.* 2003). We did not find differences in locomotor activity by HF feeding per hour in the activity-selected mice relative to when they were feeding a LF diet. However, the direction of change by HF feeding relative to LF feeding in the selected females was positive (+22.7%), whereas it was negative (-8.2%) in the control females. This caused differences in locomotor activity between selected and control females to be more significant in the HF diet condition than in the LF diet condition (Vaanholt, Jonas *et al.* 2008).

Both sexes of the activity-selected line showed increased energy expenditure relative to the control mice, particularly in selected females feeding the HF diet in the dark phase (Fig. 2). Calculation of the rates of fuel oxidation (Lusk G 1976) revealed that the increase in energy expenditure in the selected males was mainly due to increased oxidation of carbohydrates. In the selected females, however, the increased energy expenditure was mainly attributed to an exaggerated oxidation of fat, particularly when these selected females were feeding a HF diet (Fig. 4). A few points can be raised about this phenomenon. In our previous report on these mice, we observed total resistance to HF diet-induced obesity in the selected females, but not in the selected males (Vaanholt, Jonas *et al.* 2008). The resistance in the females was observed despite an increase in ingestion of the HF diet. It is likely that the resistance to dietary fat-induced obesity is caused by increased expenditure of fat. Selected males did show weight gain on the HF diet (Vaanholt, Jonas *et al.* 2008), which appears to be consistent with the fact that they spared fats by increasing carbohydrate oxidation instead. Thus, the elevated cage activity (in the absence of wheels) is fueled differently in the selected males and females by either oxidation of carbohydrates or fats respectively, and this might reflect differences in metabolic enzymatic machinery between male and female selected mice (Houle-Leroy, Garland, Jr. *et al.* 2000). Also other studies have reported sex differences in metabolic profiles with respect to physical activity in rodents (Tate & Holtz 1998) and human beings (Hackney, Muoio *et al.* 2000;Aulin 1995). The difference in fuel oxidation intuitively fits the observation that the selected males increased their

nocturnal food intake earlier than the control males did, which might have sparked carbohydrate oxidation as a result of absorbed nutrients. Female mice, both activity-selected and controls, delayed their intake during the dark phase compared to the males. Thus, to cover energetic expenses for increased voluntary activity at the beginning of the dark phase, particularly the selected females had to free-up and utilize their stored fat. This might imply that they developed an energetic deficit at the beginning of the dark phase, and that elevated food intake of the selected females relative to the controls during the ensuing light phase might be viewed as a compensation for this deficit. Similar results have been seen in humans following exercise (Finlayson, Bryant *et al.* 2009). Such deficits may have been even greater if the mice had been housed with access to running wheels, which substantially increases energy expenditure (e.g., (Swallow, Koteja *et al.* 2001;Vaanholt, Garland, Jr. *et al.* 2007).

In the present study, we performed a two-choice diet preference test just before the start of the dark phase and just before the start of the light phase. The results (Fig. 6) showed that mice feeding the LF food as their regular diet had a strong preference for the HF diet, whereas this preference was much lower when the regular food was the HF diet. The latter was particularly obvious right before the start of the dark phase, when indeed carbohydrates are generally preferred over fats, at least in rats (Shor-Posner, Brennan *et al.* 1994;Tempel, Shor-Posner *et al.* 1989). In the selected males feeding the HF diet, the relative preference for the LF diet during the late night phase over the HF diet was weaker than in the control males feeding the HF food as their regular diet, indicating that preference for carbohydrates in the selected males is lower than in the control males. In the females, however, the opposite response was observed. Instead of lowering the preference for the LF diet, there was a tendency for increased LF selection from the choice of the two diets, and this LF selection was markedly enhanced at the end of the light phase. One explanation for the increased selection of the LF diet may be that peripheral metabolic sensors inform them to do so (see (Ritter, Ritter *et al.* 1999;Singer, York *et al.* 1998)). Hence, a supply of glucose from carbohydrates is beneficial to sustain elevated physical activity. Once the animals are depleted of carbohydrates (blood glucose, stored glycogen), any intensity of physical activity will become difficult. Even though typical 'fat-burning exercise' is generally of lower intensity (i.e., fats are metabolized greatly at lower to moderate intensities of exercise), animals still need glucose to keep active without fatigue (Gomes, Rezende *et al.* 2009). So in other words, without glucose, animals run the risk of utilizing less fats as "fat burns in the flame of carbohydrate" (after Sir H. Krebs, Nobel Speech, 1953). This problem may be less severe in the activity-selected males than in their female counterparts, because their intensity of physical activity is much lower than in the females. Another, or perhaps complementary mechanism explaining this increased carbohydrate preference may be that when selected females do not sufficiently increase fat stores during the active phase, this renders plasma leptin levels lower than in controls (Vaanholt, Jonas *et al.* 2008). A relatively low plasma leptin level may increase hypothalamic levels of neuropeptide Y (Schwartz, Erickson *et al.* 1998), which in turn would stimulate consumption of carbohydrates and hence selection of the LF diet (Jhanwar-

Uniyal, Beck *et al.* 1993;Stanley, Daniel *et al.* 1985). Finally, it cannot be excluded that differences in diet selection among groups is the result of differences in reward pathways (Keeney, Raichlen *et al.* 2008).

Taken together, selected males and females have different temporal couplings of ingestive behavior and fuel oxidation, with selected females presumably more relying on fuel reserves at the onset of the dark phase, and selected males showing more anticipatory intake to cover their expenses. The stronger preference for carbohydrates in the selected females, particularly when they are adapted to feeding HF food as the normal diet, may indicate that they have difficulties providing sufficient intermediary metabolites from carbohydrate metabolism in order to utilize fats. One of the implications in the females may be that they have the risk of "missing" compensatory intake when food is suddenly not available on the next day, which might spiral the selected females sooner towards emaciation when the exaggerated spontaneous activity is not prevented. It might be speculated that some of these mechanisms are homologous to those that serve the biological underpinnings of anorexia nervosa (Sodersten, Nergardh *et al.* 2008).

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