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

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

a study in mice

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CHAPTER 1

GENERAL INTRODUCTION

Most animal species can move across the environment. This is usually brought about by muscular activity at the expense of metabolic fuels, and is general referred to as "physical activity". The level of physical activity can vary tremendously between animal species and even between animals belonging to the same species. This may, for example, depend on the environmental challenges which animals sometimes face, or the goals that animals have set out to achieve (e.g. for certain needs or rewards). Besides the level of planned or goal-oriented physical activity, the level of unplanned or "voluntary" activity can also differ largely between individuals. In human beings, for example, this may be related to the degree of fidgeting, non-specific ambulation, or other spontaneous every-day-life behavior (Levine et al. 2005; Ravussin 2005). Within individuals, levels of planned and unplanned behavior can be inversely related over some time, for example, when periodical heavy exercise is interspaced by increased resting and recovery. Besides this interdependency, it is clear that some individuals are more active than others under any condition. Evidence exists that the display of increased physical activity is a complex trait composed of several subordinate traits (John G. Swallow and Theodore Garland Jr 2005). These subordinate traits may be represented by several aspects of neurobiology, physiology, morphology, and performance, and is probably regulated by numerous genes (Garland, Jr. and Kelly 2006; Lightfoot et al. 2008; Simonen et al. 2003; Benjamin et al. 2002). In nature, the array of subordinate traits may have evolved because the cumulated trait of increased physical activity had advantageous effects on animal fitness under certain environmental conditions (Garland, Jr. and Kelly 2006). A major aim of the current thesis is to investigate differences in the subordinate traits in mice which display high and normal physical activity. A second major aim was to investigate whether and how mice with these presumed differences in subordinate traits are resistant to metabolic derangements when provided with "Western-type" unhealthy diets. This issue is extremely relevant in light of the consensus that obesity and associated metabolic derangements are the result of ingesting an unhealthy diet consisting of too much saturated fat and unrefined sugars combined with a sedentary life-style (Phelan 2009; Manfredini et al. 2009; Blair and Morris 2009).

1. Complex diseases

According to evolutionary biologists, the human genome was inherited from the paleolithic time when human life was programmed for daily physical activity and ingestion of a high-fibered diet (Eaton and Eaton, III 2000). Since then, our genome changed little, but environmental factors transformed to a large extent into the one that we are living today (i.e., lower physical activity, changes in dietary patterns but also other factors like psychosocial stress, smoking, alcohol consumption and hazardous environmental compounds). At the basis of this theory is the thrifty

genotype hypothesis which explains that genetic factors only predispose individuals to diseases but environmental factors determine phenotypic expression whether the disease becomes manifest (Neel 1999). Perhaps one of the most important factors is the increase in sedentary lifestyle, mostly caused by prolonged sitting (Rosenberg et al. 2008) Levine and colleagues found that this is associated with a reduction in non-exercise activity thermogenesis (NEAT), and might propel weight gain in a situation of overfeeding (Levine et al. 2005; Ravussin 2005). This global trend is likely to continue, given the increasing availability of personal computers, TV and transportation trends (Robinson et al. 2003; Sturm 2004). One of its detrimental effects is the loss of activity of large skeletal muscles which are known to utilize a large amount of bodily fuels (Hamilton, Hamilton, and Zderic 2007). Moreover, changes in the world food economy increased consumption of energy-dense diets high in saturated fat and high in refined carbohydrates and together with a sedentary lifestyle this obviously favors weight gain. Particularly, visceral obesity is a strong risk factor in attracting cardiometabolic disturbances such as hypertension, hypertrigliceridemia, arteriosclerosis, hyperglycemia and hyperinsulinemia (collectively called "the metabolic syndrome") that all predispose to the development of type 2 diabetes and cardiovascular diseases (Despres et al. 2008). It has been suggested that an insufficient increase in the storage capacity of white adipose tissue leading to overflow of fats in metabolic tissues and organs is responsible for the occurrence of above-mentioned diseases (Frayn 2005). Consuming a diet rich in saturated fat and/or refined sugars calls forth an excess in body fat. This further leads to elevated insulin and leptin levels, and usually is accompanied by insulin- and leptin resistance (two of the hallmark in obesity, type-2 diabetes and metabolic syndrome) in the periphery and the central nervous system. A protective factor that is down-regulated in the state of progression towards cardiometabolic disease is adiponectin (Arita et al. 1999), an adipose tissue secreted product, and its low levels are correlated with visceral obesity (Okamoto et al. 2006) and insulin resistance (Weyer et al. 2001).

2. Possible mechanism through which physical activity can help in the treatment of complex diseases

Evidence over the past decades, including epidemiological, prospective- and intervention studies has documented that physical activity, combined activity and diet interventions can relieve progression of chronic diseases or even reverse existing diseases (Donnelly et al. 2009). The degree of physical activity is positively related to improvement of cardiometabolic functions (training effect), while physical inactivity causes degeneration in many bodily functions. In the cardiovascular system, physical activity causes an increase in oxygen consumption and blood flow to meet the increased energy demands of muscular work. On the long term, physical activity increases cardiac volume, decreases heart rate and blood pressure through the mechanism of

influencing the flexibility and elasticity of blood vessels. In contrast, when these functions start to decline, cardiovascular diseases could take place, which mostly track the degree of obesity (Richardson et al. 2000). Furthermore, the increased oxygen consumption may cause a shift in substrate utilization from carbohydrates to fat oxidation, which has been highlighted to give protection against obesity and diabetes type 2 (Metz et al. 2005; Wasserman and Vranic 1986). In the same time, changes occur in the musculoskeletal system shifting muscle fiber type from fast to slow twitches which has also been shown to provide protection against obesity and type 2 diabetes (Tanner et al. 2002). Moreover, the increased aerobic metabolism, increasing plasma clearance of glucose, free fatty acids, triglycerides and sensitivity to insulin and leptin provides protection against obesity (Goodpaster and Kelley 2002). The nervous system is also stimulated since physical activity increases the numbers and intensities of connections between neurons increasing memory and learning (Dishman et al. 2006; Rhodes et al. 2003) which all provide protection against neurological disorders that has also been associated with the development of diabetes type 2 and obesity (Ristow 2004).

3. Animal studies

Numerous animal studies have focused on energy balance regulation under conditions of feeding diets differing in fat/sugar content, but much less attention has been paid to the role of physical activity. Although one can study individual difference in the display of physical activity in animals of outbred populations or in inbred strains which display large variations in it, we took the advantage of mouse lines that were selectively bred for high and normal running wheel behavior. Selective breeding uses a largely heterogeneous animal population from which lines of animals are then selectively inbred for a given character or trait of interest. Each generation of inbreeding increases the probability of attaining homozygosity at specific genetic loci resulting in a clear divergence in the phenotypic expression of the given trait (Britton and Koch 2001). Through this technique, Garland and colleagues have made selection lines of mice for high running wheel activity in four replicate lines, and four control bred lines which were not selected, in order to ascertain that observed differences are attributed to the effects of selection rather than random genetic drift (Swallow, Carter, and Garland, Jr. 1998b; T.Garland 2003). Animals selectively bred for the trait of high voluntary wheel running activity ran 2.7 times more than mice from the randomly bred control lines already in generation 16, and this difference was sustained throughout the following generations (T.Garland 2003). In addition, they ran with higher velocities (Swallow, Carter, and Garland, Jr. 1998a) and more intermittently in shorter bouts (Girard et al. 2001). The running-wheel selected mice were also more active in their homecages when deprived from wheels (Rhodes et al. 2001) relative to controls. In this thesis, I used two of the replicate lines selectively bred for high wheel running activity (lab designated line 7 and line 8) that showed the most phenotypic characteristics and one of their randomly bred control lines

(line 2). Animals were obtained from generation 31 in chapter 3, generation 49 in chapter 2,5,6,7 and generation 52 in chapter 4.

Artificial selection is thus a tool to investigate in the laboratory line differences in a specific characteristic which originated from certain individuals in the population (Landgraf and Wigger 2002). As expected, differences in the trait of running wheel activity results in alterations of subordinate traits, as has mentioned in previous studies revealing several specific differences in physiology and morphology between selected and control lines differing in the level of wheel running activity. Active lines have smaller body masses, lower body fat percentage, increased food intake, low plasma levels of leptin and elevated plasma levels of adiponectin (Vaanholt et al. 2008; Vaanholt et al. 2007) relative to control lines, and the differences become particularly striking when they are subjected to a high fat diet (Vaanholt et al. 2008). Furthermore, their hind limb muscles have reduced mass, have an increase in oxidative fibers, in mitochondrial enzyme activity, in mass-specific capillarization, in glycogen storage and in muscle glucose transporter (GLUT-4) number (Gomes et al. 2009; Wong et al. 2009; Guderley et al. 2008). These physiological/morphological changes may be viewed as "adaptive" to sustain high physical activity levels.

The occurrence of trait-differences may appear not only in physiology and morphology, but may also be expressed in emotionality/personality (Heinrichs and Domes 2008; Wolf et al. 2007). In general, animals can cope actively in psychological stressful situations with low emotional reactivity characterized by impulsiveness, or they cope passively with high emotional reactivity characterized by anxiety. These two emotional and behavioral characteristics have been shown to be associated with specific neuroendocrine and physiological profiles (Steimer, la Fleur, and Schulz 1997). The inter-individual differences in these profiles and emotional behaviors in any animal and human population could be critical in regard of adaptive responses and capacity to challenging situations and also in resistance or susceptibility to certain stress-related pathologies (McEwen and Stellar 1993). Furthermore, this could depend on the type of diet which is eaten. For example, a palatable high fat/high sugar diet is known to have "psychological stress"-relieving properties compared to the situation when a healthy, but less palatable diet is eaten (la Fleur et al. 2007; van Dijk and Buwalda 2008). In turn, the expression of these psychological stress relieving actions of certain diets might depend on the subordinate traits for emotionality/personality which animals occupy. In the present thesis, differences in these emotionality/personality styles were studied in addition to the physiological/metabolic profiles in order to better understand and characterize mice with high activity levels versus those with normal activity levels.

4. Early life experience

Not only adult life-style factors, but nutritional factors during the perinatal stage could predispose an individual towards obesity and other associated diseases such as type 2 diabetes, metabolic syndrome (Levin 2006; Parente, Aguila, and Mandarim-de-Lacerda 2008). This could particularly occur when the availability of energy dense foods are high during perinatal stage, resulting a programming effect on neuroendocrine system and hypothalamic feeding circuits later in life. These are stated in the theory of "Developmental Origins of Adult Disease" or "developmental programming" and in the "thrifty phenotype" hypothesis (Hales and Barker 2001; Barker 2004). This can be studied in laboratory animals either by changing the quantity of energy availability to offspring with altering litter size or by changing the quality of maternal diet through supplying pregnant and lactating mothers with a high energy dense diet, rich in saturated fats and sugars (Plagemann 2005; Plagemann 2005; Armitage, Taylor, and Poston 2005). In both ways, susceptibility to metabolic diseases can be affected in adult offspring due to early programming.

Since the perinatal environment is a strong determining factor in the development of offspring energy balance and behavior later in life, a third major aim of the current thesis was to investigate the influence of the perinatal environment on development of the trait of high physical activity. For this reason, pregnant mice from control and selection lines were submitted to a healthy fibered low-fat diet, or to a "western type" high fat diet. Furthermore, the relevance of the post-natal period was investigated by cross-fostered pups between mothers from control and active lines to follow up their energy homeostasis and behavior later in life. The procedure of "cross-fostering" needs to be performed right after birth when the biological offspring is removed from their mother and placed with a surrogate mother (McGowan, Meaney, and Szyf 2008). In general, cross-fostering is used to study the impact of the postnatal environment on gene expression as well as on behavioral patterns. When cross-fostered offspring show similar traits as their biological parents but different than their foster parents, the trait is considered to have a mixed genetic and prenatal basis. Contrary, when the offspring develops a trait different than their biological parents and similar to their foster parents, the environmental factors are considered to be dominant. However, in most of the cases, there is a blend of the two, which shows both genetic and environmental influences at play.

5. Aim and outline of this thesis

Since selective breeding for a complex trait causes several associated physiological, morphological and psychological changes, it may also be regarded as an appropriate tool to study complex diseases. With respect to obesity and the associated metabolic syndrome, the trait of high

physical activity clearly causes resistance to these phenomena. The use of mice selectively bred for increased physical activity can therefore facilitate a better understanding of aetiology and prevention of obesity and associated metabolic derangements at adulthood, as well as during perinatal development.

Chapter 2 is aimed at studying behavioral consequences of selective breeding for high voluntary wheel running activity for anxiety, exploration and learning. Circadian patterns of energy balance-related factors such as food intake, carbohydrate-, fat oxidation, energy expenditure and also running wheel activity were studied in relation to food preference in presence of normal or 60% high fat diet feeding in **chapter 3**. **Chapter 4** is aimed to study effects of high fat-high sugar diet feeding on energy balance, stress related behavior and corticosterone levels. **Chapter 5** focuses more on perinatal implications, where perinatal effects of high fat-high sugar diet feeding is described on maternal energy regulation and litter characteristics in relation litter size at pre-weaning stage. **Chapter 6** presents the consequences of the perinatal high-fat-high sugar diet feeding on the offspring energy balance later in life. The last experimental chapter (**chapter 7**) is aimed to study effects of litter size equalization to 10 pups/litter and effects of cross-fostering on maternal energy regulation and offspring energy balance at pre- and post-weaning periods. Finally, general conclusions and implications of the results presented in this thesis are discussed in **chapter 8**.

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CHAPTER 2

PERSONALITY TRAITS ARE AFFECTED BY SELECTIVE BREEDING FOR INCREASED WHEEL-RUNNING BEHAVIOR IN MICE

I. Jónás, K. A. Schubert, F. Reijne, J.Scholte, T. Garland, Jr, M. P. Gerkema, A. J. W. Scheurink, C. Nyakas and G. van Dijk

Summary

Voluntary physical activity may be related to personality traits. Here, we investigated these relationships in two mouse lines selectively bred for high voluntary wheel-running behavior and in one non-selected control line. Selection lines were more explorative and "information gathering" in the open-field test, either with increased upright positions or horizontal locomotion toward the middle ring. Furthermore, one of the selection lines had an increased risk-taking behavior relative to the control line in approaching a novel object placed in the center of the open field. However, anxiety behavior was increased in selection lines during the plus-maze test. Maze learning was not statistically different among lines, but routine behavior was increased in both selection lines when the maze exit after two days of testing was displaced. Specifically, in the displaced maze, selected mice traveled more frequently to the old, habituated exit, bypassing the new exit attached to their home cage. The increased routine and exploratory behavior may be adaptive to sustain high activity levels.

1. Introduction

Much attention has been paid to the mechanisms by which physical activity helps to avoid or ameliorate obesity and associated metabolic derangements (Brock, King *et al.* 2005;Hayes & Kriska 2008;Levin & Dunn-Meynell 2004;Patterson & Levin 2008). In addition, evidence is increasing that physical activity improves mental health (Dey 1994;Dietrich & McDaniel 2004;Hillman, Erickson *et al.* 2008). For example, frequent daily performance of exercise, such as endurance running, is able to reduce levels of anxiety and depression (Antunes, Stella *et al.* 2005;Kligman & Pepin 1992), and to augment learning capability in human beings (Hillman, Erickson *et al.* 2008). Homologous beneficial effects of physical activity have been demonstrated in rodents on energy balance regulation (Bi, Scott *et al.* 2005;Bell, Spencer *et al.* 1995), but also on emotionality, stress coping, and learning (Greenwood, Strong *et al.* 2007;Pietropaolo, Sun *et al.* 2008;van Praag, Christie *et al.* 1999;Rhodes, Van *et al.* 2003).

In a meta-analysis of Rhodes and Smith, it was found that the amount of voluntary or spontaneous physical activity in humans was positively related to such major personality traits as increased extraversion and conscientiousness, and negatively to neuroticism (Rhodes & Smith 2006). A venue not thoroughly explored is the possibility that the display of voluntary physical activity and certain personality traits have common neurobiological or endocrine mechanisms. One approach to investigate whether and how personality traits are associated with the level of spontaneous physical activity is to study these in mice from lines that have been selectively bred for high voluntary wheel-running behavior (Swallow, Carter et al. 1998;Garland, Jr. 2001) and compare these to non-selected control mice. If certain personality traits are related to voluntary physical activity, then one might hypothesize that the expression of these personality traits is amplified or diminished in mouse lines selected for high voluntary physical activity.

After selective breeding for 16 generations, the four replicate activity-selected lines of mice ran approximately 2.7 times more revolutions in their running wheels per day compared to four non-selected control lines (Swallow, Carter *et al.* 1998), mainly by running at higher speeds rather than for more time per day. They also ran more intermittently, with shorter and more-frequent bouts (Koteja, Garland, Jr. *et al.* 1999;Girard, McAleer *et al.* 2001). After locking the wheels, the high-runners spent more time climbing the locked wheels, apparently trying to run (Koteja, Garland, Jr. *et al.* 1999). When housed in cages without wheels, high-runner mice exhibited elevated home-cage activity (Rhodes, Hosack *et al.* 2001;Malisch, Breuner *et al.* 2008;Rhodes, Hosack *et al.* 2001).

Despite a large difference in the level of chronic wheel running between selected and control mice, Rhodes et al. only found increased learning capabilities (following chronic wheel access), in the control lines (Rhodes, Van *et al.* 2003), and this despite differences in hippocampal neurogenesis. Furthermore, Bronikowski et al. did not observe differences in exploratory behavior between selected and control lines in a standard open-field test (Bronikowski, Carter *et al.* 2001). Although these data could be interpreted as evidence against a "personality traits

hypothesis of voluntary behavioral arousal", they did, however, find that selected mice displayed fewer turns in their travel paths, which could be indicative of increased motivated behavior in these animals. Finally, selected and control mice differ with respect to stress coping behavior, with the latter showing more behavioral despair during forced swimming (Malisch, Breuner *et al.* 2009), and less predatory aggression towards crickets (Gammie, Hasen *et al.* 2003).

In the present study, we re-evaluated the hypothesis that mice selectively bred for high voluntary wheel running activity have certain personality traits that differ from controls. We tested this hypothesis by analysing a broad spectrum of behaviors, including those that reflect anxiety, exploration, learning, and routine behavior in two activity-selected lines and one control line.

2. Materials and methods

2.1. Animals

In July-August 2006, mice from generation 45 of the selection experiment for high voluntary wheel running (Swallow et al., 1998) were shipped from The University of California (Riverside, CA) to the University of Groningen (Haren, the Netherlands). Mice used in the present study were offspring of these individuals. For a detailed description of the selection experiment, see (Swallow, Carter *et al.* 1998). In brief, the base population was Hsd:ICR mice, an outbred stock with relatively high levels of genetic variation. In the original selection protocol, eight lines of mice were created (4 selected and 4 controls). From this we used male and female mice from two selection lines (lab designations line 7 and line 8) and one control line (line 2). Thus, for simplicity, we did not study selection lines 3 and 6, both of which exhibit the "mini-muscle" phenotype, caused by a Mendelian recessive allele with many pleiotropic effects (Garland, Jr., Morgan *et al.* 2002;Hartmann, Garland, Jr. *et al.* 2008). Because we studied only two selection lines and one control line, the generality of our results would need to be confirmed in future studies of all eight lines in the selection experiment.

For the present study, mice were housed in same-sex groups of two or three per cage, with *ad libitum* food (Standard lab chow RMH-B 2181, HopeFarms BV, Woerden, NL) and water, at an ambient temperature of 22±1 °C, and maintained on a 12:12 light-dark cycle with lights on (100 Lux) at 8am. Wood shavings and EnviroDry® were used as bedding material. Weaning occurred at 21 days of age. 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. Experimental design

After a habituation phase, two-month-old male (6-8 per line) and female mice (6-8 per line) were subjected to an open-field test, elevated plus-maze test, and complex maze learning tests on

different days in the order as mentioned. Each experiment was performed during the beginning of the light phase, at a time when the general behavioral activity is rather low (Malisch, Breuner *et al.* 2008), and effects may not be dampened by the noise of general behavioral arousal towards the end the light phase or dark phase (van Dijk & Strubbe 2003). Before each behavioral test, animals were brought in their own cage to a dimly illuminated room (10 Lux) adjacent to the experimental room 30 min. prior to the behavioral test to reduce the level of psychological stress associated with the transport. The light intensity in the experimental room ranged between 70 and 80 Lux. Each time before testing, the open field, plus maze, and complex maze were rigorously cleaned with soap and water, and investigators always left the room during the test. Behavior was recorded (Sony Handycam), and later analyzed using Eline software (dr F. Maes, University of Groningen).

2.2.1. Open-field behavior

Explorative and spontaneous locomotor activity were observed in a circular horizontal arena. In this arena (ø 1.0 m, height of vertical surrounding wall: 0.4 m), concentric circles were depicted at one-third and at two-third of the arena's diameter, dividing the surface into a center, a middle ring and an outer ring (adapted from (Walsh & Cummins 1976)). At the beginning of each trial, the mouse was placed in the center of the arena.

Mice were tested for 5 min, including 1 min at the end when a 1-kilogram brass weight was introduced into the center of the open field to test the animals' exploratory behavior for a novel object. Behavior was recorded and the following traits were determined: percentage of time spent in outer ring, middle ring and center, number of visits into each ring (visit frequency), total number crossings from one ring to the next (crossing frequency), and number of rearings.

2.2.2. Elevated plus-maze

Anxiety was evaluated using the elevated plus-maze test, which is based on the aversion for open, well-illuminated spaces (Treit, Menard *et al.* 1993). The elevated plus-maze consisted of two open arms and two closed arms facing each other, 40 cm above the floor. Each arm was 29 cm long and 5 cm wide. The closed arms had 15 cm high walls with a closed top. At the beginning of each trial, the mouse was placed on the central area (5 x 5 cm) facing an open arm and allowed to explore the maze for 5 min. The following traits were determined: time spent in open arms and closed arms, time spent in central area, number of visits to open arms and closed arms, and number of total arm entries. An arm entry was counted when the front paws were placed on the arm.

2.2.3. Complex maze

Spatial learning performance was assessed with a complex maze that was based on the configuration originally developed by Rabinovitch and Rosvold (Rabinovitch & Rosvold 1951), and adapted for mice. The maze was constructed of a horizontal surface (25 x 19 cm), with

vertical barriers (7 cm high) arranged to form a maze (see figure 1). To prevent the mouse from jumping out, the maze was enclosed in a transparent Plexiglas box (which allowed observation of the mouse's behavior).

Figure 1. The complex maze adapted from Rabinovitch and Rosvold (1951), with the entry on the left hand side and the exit on the right.

From the habituation room, a mouse inside its home cage was brought into the experimental room, and the cage was connected via a Plexiglas tube (ø 5 cm, length 17 cm) to the maze entry (left hand side in figure 1). After the mouse entered the tube towards the maze, a sliding door inside the tube was closed, and the home cage was disconnected and reattached to the exit tube on the opposite corner at the right hand side of the maze (see figure 1). Then the investigator left the room, and followed the mouse's behavior via a monitor outside the experimental room. Once the mouse found its home cage again, the investigator entered the room and returned the mouse, inside its cage, to the adjacent room. On the day prior to the test, mice were once allowed to explore the maze. On two successive experimental days, mice were subjected to the maze four times a day with sessions 30 min apart. In all trials, the latency of reaching the exit and the number of errors were scored. On the third day, mice were once tested in the maze, but with the entrance and exit laterally inverted (figure 2b). It was assessed whether and how long it took for the mice to reach the "new" displaced exit, and whether they exited the maze directly through the "new" exit, or searched the corner of the old exit first.

2.3. Statistical analysis

Data for open-field and elevated plus maze were analysed with GLM models in SPSS (version 12). Line and sex were added as fixed factors. Further individual comparisons were performed using Tukey's post-hoc tests. In the complex maze analysis, the GLM models for repeated measures were applied. When the entrance and exit of the maze were inverted laterally on the third day, data were further analyzed by two-tailed t-tests and Chi-squared test for nominal data. In the latter, we compared the number of mice going directly to the inverted exit and the ones that were still looking at the old position. Results are given as means ± SEM and are considered significantly different when $p<0.05$.

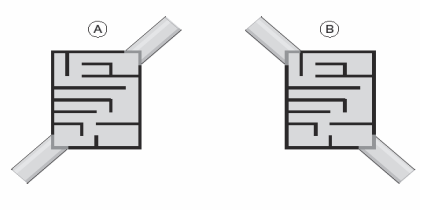

Figure 2. Configuration of the complex maze during the first two days (a), and during the last trial on the third day (b), where the entrance and exit were laterally inverted. Note that the on the third day the mouse bypasses the "new" displaced exit (configuration b) when searching for the "old" exit (configuration a).

3. Results

3.1. Open field

During the first four minutes, multivariate analysis including all test variables revealed effects of line ($F(14,116)=3.89$; $p<0.001$), but not of sex or of the interaction between line and sex. Specifically, the percentage of time spent in different compartments was significantly influenced by line ($F(2,53)=6.20$; $p=0.004$); post-hoc analysis revealed that line 8 mice spent significantly more time in the middle ring than line 7 ($p=0.004$) and line 2 mice ($p=0.05$) (figure 3, top panel). Furthermore, a line effect was also found in the number of visits in the outer ($F(2,53)=4.89$; $p=0.01$) and middle rings ($F(2,53)=5.34$; $p=0.008$). Line 8 had a higher frequency of middle-ring visits than line 2 ($p=0.01$), and line 8 had higher frequency of outer- ($p=0.01$) as well and middle-ring ($p=0.04$) visits than line 7 (figure 2, middle panel). The number of rearings also revealed an effect of line ($F(2;53)=14.21$; $p<0.001$); line 7 had a higher number of rearings than line 2 ($p<0.001$) and line 8 ($p<0.001$) (figure 3, lower panel).

Upon introduction of the novel object in the center of the open field (during the last minute), multivariate analysis did not show any significant effects. However, univariate analysis revealed effects of line on the time spent in the center ring ($F(2,53)=4.87$; $p=0.01$). Line 8 mice spent more time in the center than line 2 ($p<0.05$) and line 7 mice ($p=0.01$). The time spent in the outer ring tended to be influenced by line ($F(2,53)=3.14$; $p=0.05$); specifically line 8 spent significantly less time in the outer ring than line 7 ($p=0.05$).

3.2. Plus-maze

Plus-maze behavior of mice from the different breeding lines is shown in figure 4. Multivariate analysis revealed a significant effect of line ($F(12,118)=4.07$; $p<0.001$). Univariate analysis revealed the following. Firstly, an effect of line was found in the percentage of time spent in the

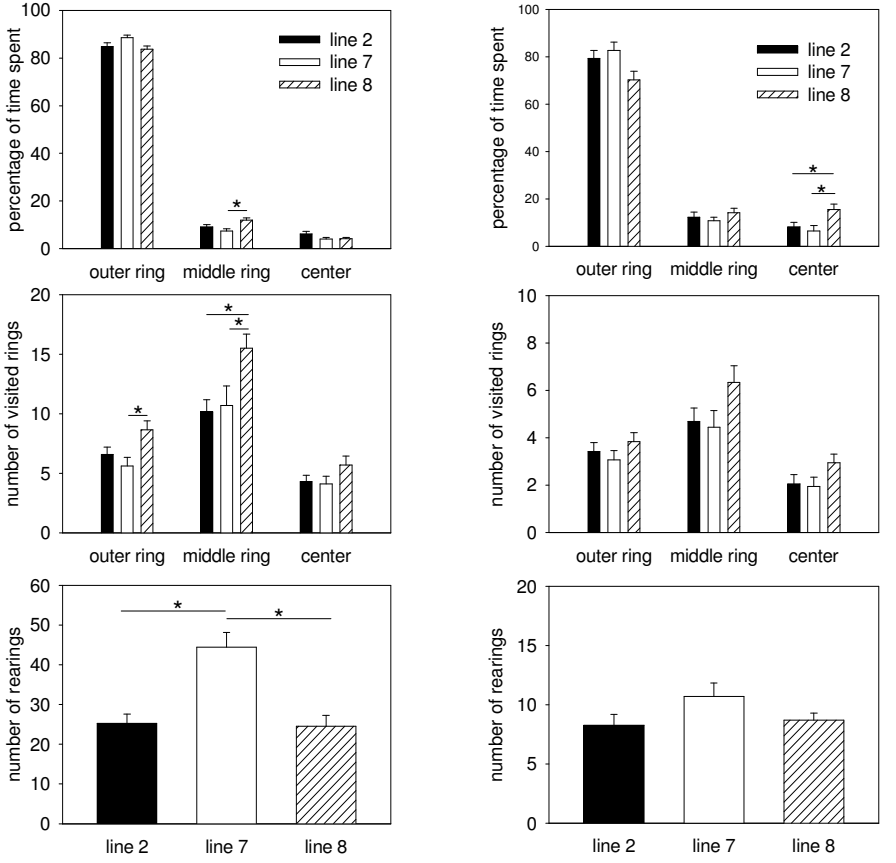

Figure 3. Open-field behavior of mouse lines 2 (control), 7 (selectively bred for high voluntary wheel running), and 8 (also selected) during the first 4 minutes (left panels) and during the last minute after which a novel object was introduced in the center of the open field (right panels). The top panels show the percentage of time the animals spent in the different areas, the middle panels show the number of visits to the different areas, and the lower panels show the total number of rearings. * indicate significant difference between means at $p<0.05$.

open arms ($F(2,54)=6.12$; $p=0.004$) and in the closed arms ($F(2,54)=6.46$; $p=0.003$). Specifically, line 8 mice spent less time in the open arms ($p=0.003$) and more time in the closed arms than line 2 mice ($p=0.003$) (figure 3, top panel). Secondly, an effect of line was also detected in the number of open arm entries ($F(2,54)=4.25$; $p=0.02$), closed arm entries ($F(2,54)=10.57$; $p<0.001$) and central area entries ($F(2,54)=7.23$; $p=0.002$). Specifically, line 8 mice made fewer entries into the open arms than line 2 ($p=0.04$) and line 7 mice ($p=0.04$); line 7 mice had higher number of

entries into closed arms relative to line 2 ($p<0.001$) and line 8 ($p=0.008$) and into the central area relative to line 2 ($p=0.01$) and line 8 ($p=0.002$). A line effect was also found in the percentage of open-arms entries ($F(2,54)=6.92$; $p=0.002$) and the percentage of closed-arms entries ($F(2,54)=6.92$; $p=0.002$); i.e., line 8 had smaller percentage of open arms entries than line 2 ($p=0.002$), and line 2 had smaller percentage of closed arms entries than line 7 ($p=0.04$) and line 8 ($p=0.002$) (figure 3, bottom panel). The total number of arm entries was also influenced by line ($F(2,54)=6.02$; $p=0.005$); i.e., line 7 had significantly more total arm entries than line 8 ($p=0.004$) and line 2 ($p=0.06$). Finally, a sex effect, but no interaction effect, was found in the percentage of time spent in the open ($F(1,43)=4.21$; $p=0.046$) and closed arms ($F(1,43)=6.30$; $p=0.015$). Specifically, females spent more time in open arms and less time in closed arms than males (individual data for females and males not shown).

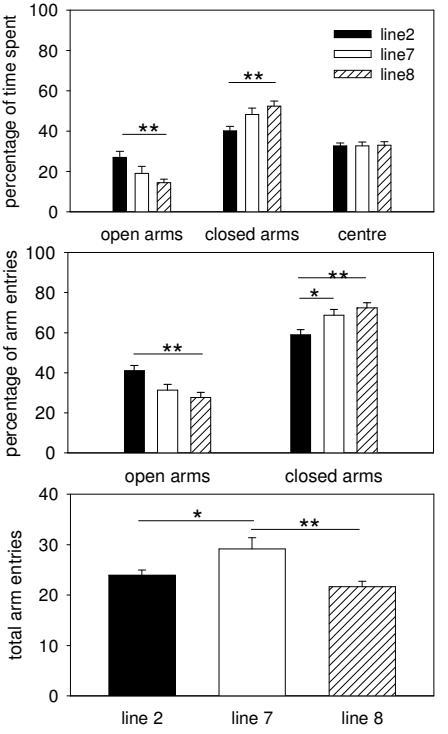

Figure 4. Plus-maze behavior of mouse lines 2 (control), 7 (selectively bred for high voluntary wheel running), and 8 (also selected). Top panel shows the percentage of time spent in the open arms, closed arms and in the center. Middle panel shows the percentage of arm entries into the open and closed arms. Lower panel shows total arm entries. Asterisks indicate significant difference between means at $p<0.05$ (*), and $p<0.01$ (**).

3.3. Complex maze

With GLM repeated-measures analysis, the performance of animals in all groups improved upon training in our complex maze. Repeated task exposure resulted in a decrease of the time spent in the maze to find the exit when observed 4 trials per day for 2 days ($F(7,43)=8.59$; $p<0.001$) (see figure 5). A significant line effect was also found ($F(2,49)=4.15$, $p=0.02$), but no sex or line*sex interaction was observed. Post-hoc analysis revealed that line 8 mice took significantly more time in the maze, particularly on day two, to reach the exit than line 2 did ($p=0.02$).

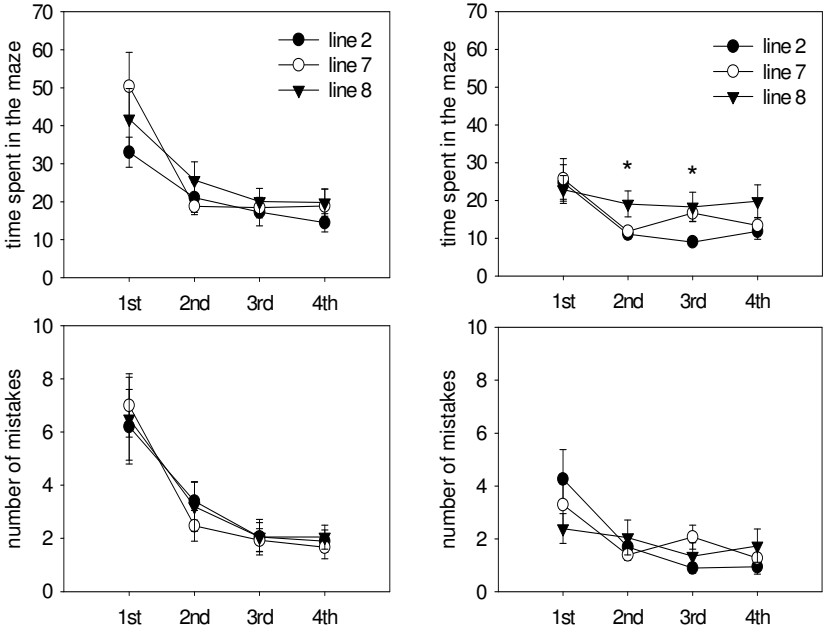

Figure 5. Complex maze behavior of mouse lines 2 (control), 7 (selected), and 8 (selected) on first day (left panels), and on the second day (right panels). Top panels show the duration in the maze to find the exit, and the lower panels show the number of mistakes the mice made. * indicate significant difference between means at $p<0.05$.

When the exit and entrance of the maze were displaced laterally, repeated measures within subject analysis revealed that mice needed significantly more time to find the new and correct exit ($F(1,55)=22.53$; $p<0.001$). Furthermore, interaction effects of time*line ($F(2,55)=3.69$; $p=0.03$) and time*sex ($F(1,55)=6.09$; $p=0.02$), but no interaction of time*line*sex were revealed, meaning that line 7 needed significantly more time to find the new exit, particularly females. This is consistent with the time spent in the maze in this last trial when the maze was

displayed laterally, showing an effect of line ($F(2,54)=4.22$; $p=0.02$); line 7 spent significantly more time to find the correct displaced exit relative to line 2 ($p=0.02$) (Figure 6, upper panel). The number of mistakes did not differ between groups (Figure 6, middle panel). Furthermore, logistic analysis revealed an effect of line ($\chi^2(2)=7.61$, $p<0.05$), but no effect of sex or line*sex interaction in the number of animals for which the first attempt led to the correct exit. Specifically, line 2 animals more frequently chose the new inverted exit than line 7 ($\chi^2(1)=7.51$, $p<0.01$) and line 8 mice ($\chi^2(1)=7.51$, $p<0.01$) for their first attempt.

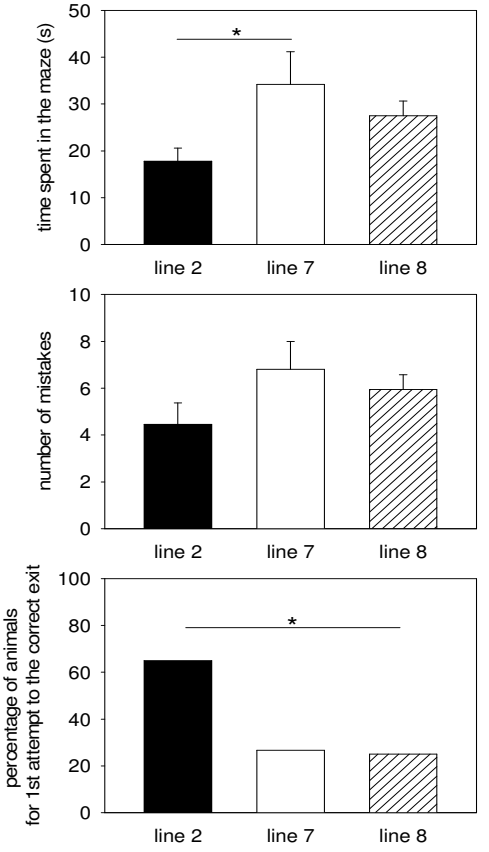

Figure 6. Complex maze behavior of mouse lines 2 (control), 7 (selected), and 8 (selected) during the final trial on the third day during which the entrance and exit were inverted laterally. Top panel shows the time spent in the maze. The middle panel shows the number of mistakes the mice make in the maze, and the lower panel shows the percentage of mice going directly at the first attempt to the new inverted exit. * indicate significant difference between means at $p<0.05$.

4. Discussion

Long-term selective breeding for high voluntary physical activity in animals may alter personality traits from certain behavioral domains that are adaptive for sustaining a high activity level. In the present study, we investigated potential differences in personality traits of two lines of mice selectively bred for high voluntary wheel-running activity and a non-selected control line. Outcomes of an open-field test (to asses explorative behavior), a plus-maze test (to assess anxiety levels), and a complex maze (to assess learning and routine behavior) indeed showed profound differences between mice of the high-activity lines and the control line. However, the magnitude and direction in the shifts of behavior in the high-activity mice relative to controls were not always uniform.

Important for consideration of the results of the present study is the fact that mice were tested in their "sedentary" state and in fact were never given access to running wheels. The reason for this was that previous exposure to wheel running may influence outcomes of behavioral tests (e.g., (Rhodes, Van *et al.* 2003;Malisch, Breuner *et al.* 2009)). Thus, here we deal with animals which normally show different amounts of locomotor behavior in running wheels, but have no possibility to express this behavior. From offspring of this generation used for other purposes, however, we know that the average wheel-running behavior in line 7 and line 8 mice was, respectively, 2 times and 2.1 times more than that of controls (unpublished results). Even without running wheels, Malisch et al. found that the selected mice have increased spontaneous locomotor behavior assessed with passive infrared detection (Malisch, Breuner *et al.* 2008;Malisch, Breuner *et al.* 2009). Increased spontaneous activity in mice might manifest itself as increased locomotion in the open field, which we scored as "exploratory behavior". There were, however, differences between the activity-selected lines. Line 7 mice showed more rearing or upright behavior, whereas line 8 mice showed more exploratory behavior by locomotion towards the middle ring (figure 3) compared to control mice. In addition, line 8 showed more exploration in the center when the novel object was placed in the center of the open field. Thus, the two selection lines expressed different "information gathering" behavior in the empty open field, and also differed with respect to "approach" and "risk-assessment" behavior (Augustsson & Meyerson 2004) when the novel object was introduced.

In the plus-maze test, selected lines and in particular line 8 (with line 7 as the intermediary phenotype) spent less time in the open arms than the control line, which is indicative of higher anxiety levels in selected than control mice. In the complex maze test, all mice showed learning performance over time. Over the course of several trials, we did not observe that activity-selected mice learned faster or slower than controls. This seems consistent with the findings by Rhodes et al, showing no differences in learning capabilities of selected mice in a Morris water maze (Rhodes, Van *et al.* 2003) compared to controls when they did not have access to running wheels. However, the selected mice from line 8, needed slightly more time to find the exit, but without making more mistakes than controls. Perhaps line 8 mice are more cautious and slower in their travel paths in "non-surveyable" environments, which may fit the

higher anxiety levels of these animals in the plus-maze test. In the "surveyable" open field, however, line 8 mice displayed more exploratory behavior and risk-assessment towards the novel object placed in the center. When the exit of the maze was displaced on day three, selected mice - and especially the females - spent longer time in the maze searching for the new exit, and they more frequently visited the original exit relative to controls. Assuming that there are no differences in visual or odor detection among lines (i.e., they all bypass the tube directly connected to their home cage on their way to the old exit), this points to a more routine-like behavior in both selected lines of mice compared to controls, irrespective of sex.

Based on the present results, it may be concluded that selection for increased wheel-running behavior has caused changes in a number of personality traits. Perhaps the increased routine-like behavior of selected mice may help to sustain increased "monotonous" physical activity, such as wheel running behavior. But what about the other behaviors, such as cautiousness, anxiety, and exploratory behavior? From an evolutionary standpoint, it may be speculated that individuals that display a high level of physical activity have an advantage in habitats that require relatively large home ranges or territories (for example, for food and/or partner seeking), provided that they are cautious, exploratory, and stick to routines at the same time. In other words, individuals ranging over relatively large areas have an elevated risk of predation, which could be compensated by appropriate behaviors (Clobert, Opplige *et al.* 2000). Correlated responses to selection can also occur because of pleiotropic gene action, which may be especially common for behavioral traits. In this event, one needs to differentiate between selection that may act to favor particular combinations of traits (i.e., caution with wide ranging, but also potentially neuroendocrine control pathways that allow increased fuel oxidation (Vaanholt, Jonas *et al.* 2008) and secondly, the response to selection, which may "drag along" any genetically correlated trait in the population at that time (see for example (Bult & Lynch 2000)), whether that genetic correlation is caused by linkage or pleiotropy. In the latter case, these interactions could be adaptive or even maladaptive (Malisch, Breuner *et al.* 2008;Malisch, Breuner *et al.* 2009). It would be of interest to test these interactions in mice living in natural habitats (Benus, Bohus *et al.* 1991).

It may seem difficult to combine the aforementioned antagonistic behaviors from a neurobiological standpoint. In their transient hypofrontality hypothesis (Dietrich 2003), Dietrich et al. suggested that there is a temporary inhibition of neural activity in the prefrontal lobe during monotonous, automated movements, such as running (Dietrich & Sparling 2004). At the same time, when neuronal activity in the prefrontal lobes become reduced, the basal ganglia are being activated, which control routine behavior (Dietrich & Sparling 2004;Graybiel 2008). It may be speculated that mice from the selected lines have a higher capacity of re-allocating neuronal activity from prefrontal cortical regions to the basal ganglia during exercise, which may help them to sustain this behavior. This may provide a neurobiological explanation for the observation that selected animals, after extensive maze-learning, visited the former "habituated" exit in the complex maze. In a novel environment, however, where the selected mice are faced with

uncertainty (such as in the open field), this switch between neuronal activity from prefrontal cortex to basal ganglia probably does not occur. If anything, the selected animals show increased curiosity and exploration in a novel environment, which reduces uncertainty and vigilance (Berlyne 1966;Dember & Earl 1957). It may be speculated that in the natural habitat, where natural resources sometimes become transiently smaller and/or predation pressure is increased, the behavioral characteristics of increased voluntary activity associated with - or perhaps causal to - curiosity and exploration may be adaptive and may increase the chances of survival.

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CHAPTER 3

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.

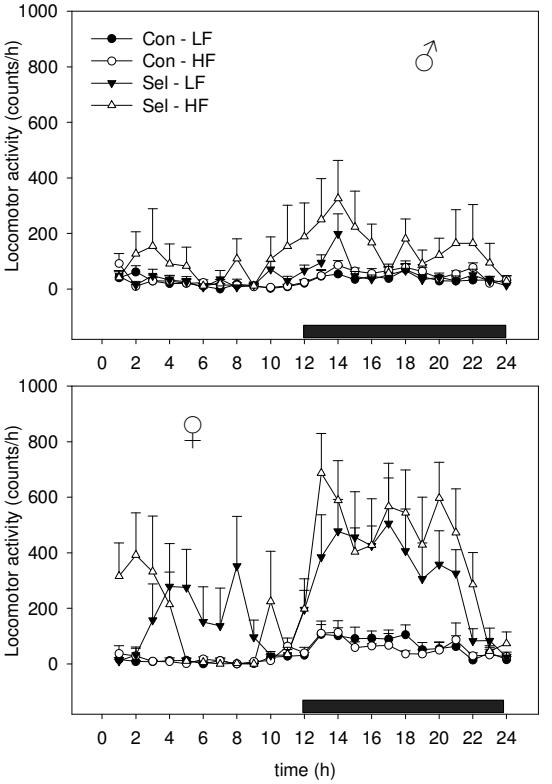

Figure 1. Locomotor activity during indirect calorimetry in control (Con) and selected (Sel) male (upper panel) and female (bottom panel) mice feeding a low-fat (LF) or high-fat (HF) diet. Dark phase is indicated by the black horizontal bar at the bottom of the graph.

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.

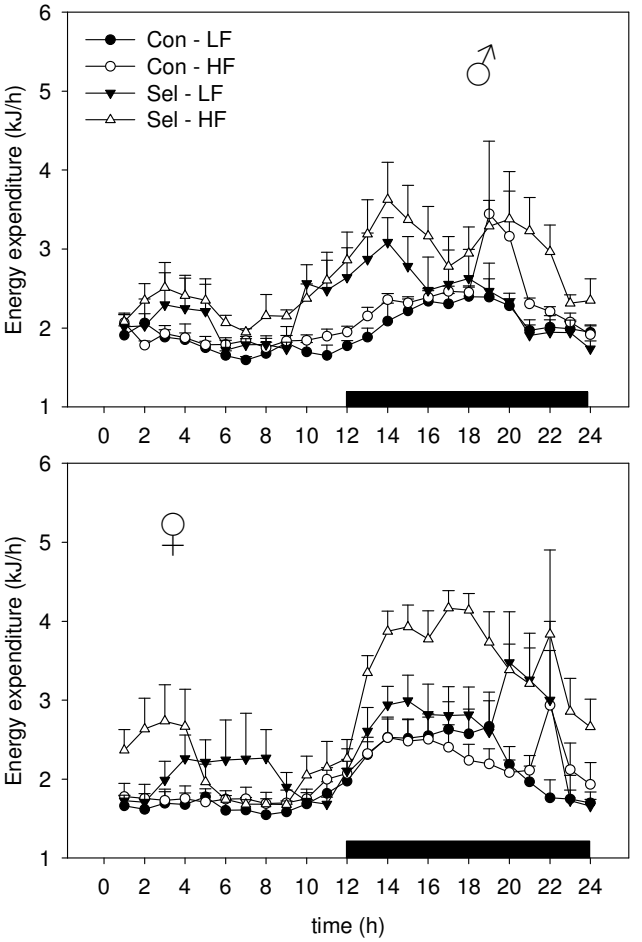

Figure 2. Energy expenditure in control (Con) and selected (Sel) male (upper panel) and female (bottom panel) mice feeding a low-fat (LF) or high-fat (HF) diet. Dark phase is indicated by the black horizontal bar at the bottom of the graph.

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.

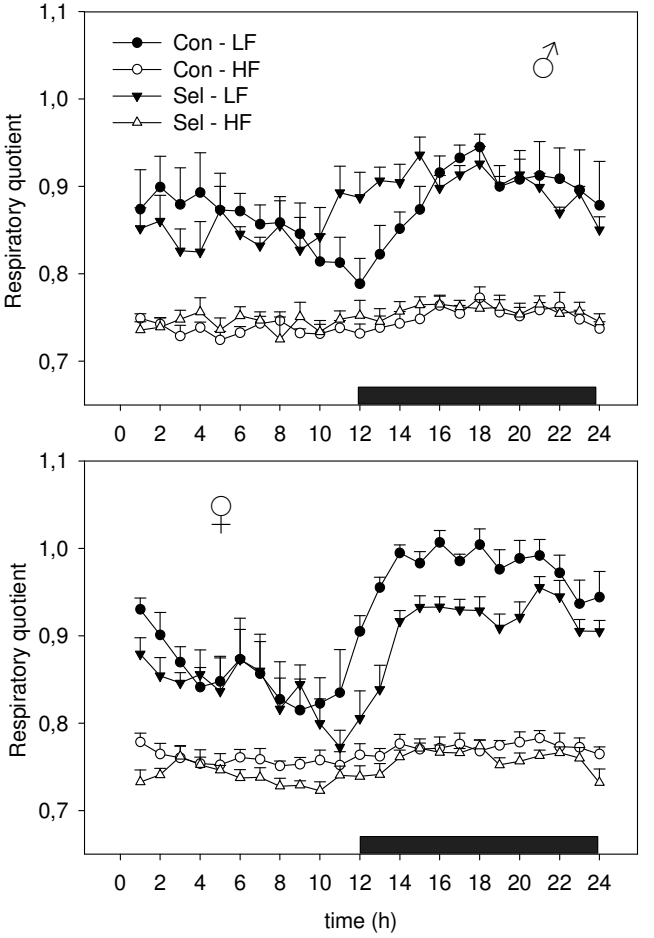

Figure 3. Respiratory Quotient (RQ) values during indirect calorimetry in control (Con) and selected (Sel) male (upper panel) and female (bottom panel) mice feeding a low-fat (LF) or high-fat (HF) diet. Dark phase is indicated by the black horizontal bar at the bottom of the graph.

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.

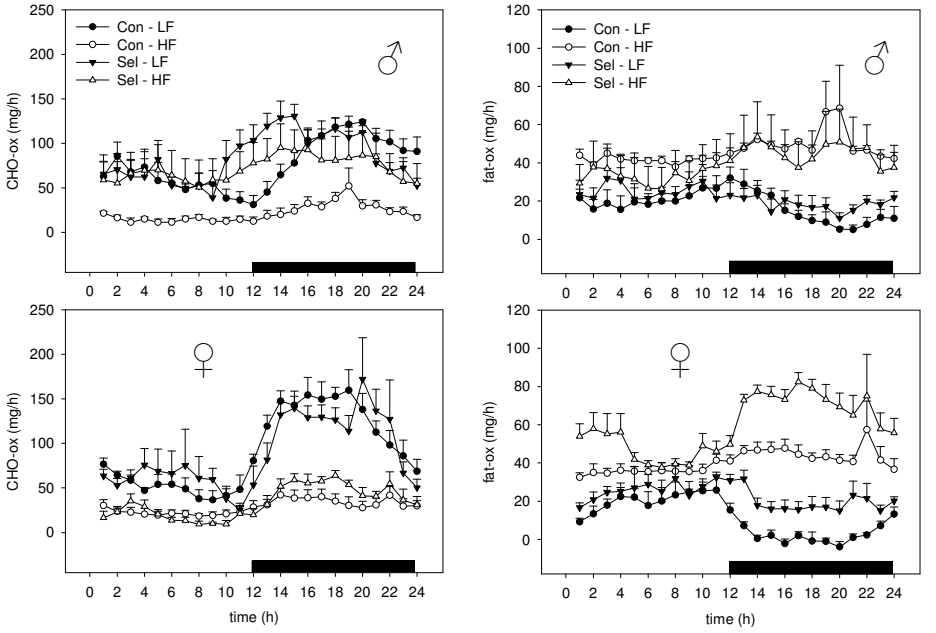

Figure 4. Calculated carbohydrate (CHO; left graphs) and fat (right graphs) oxidation in control (Con) and selected (Sel) male (upper panel) and female (bottom panel) mice feeding a low-fat (LF) or high-fat (HF) diet. Dark phase is indicated by the black horizontal bar at the bottom of the graph.

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.

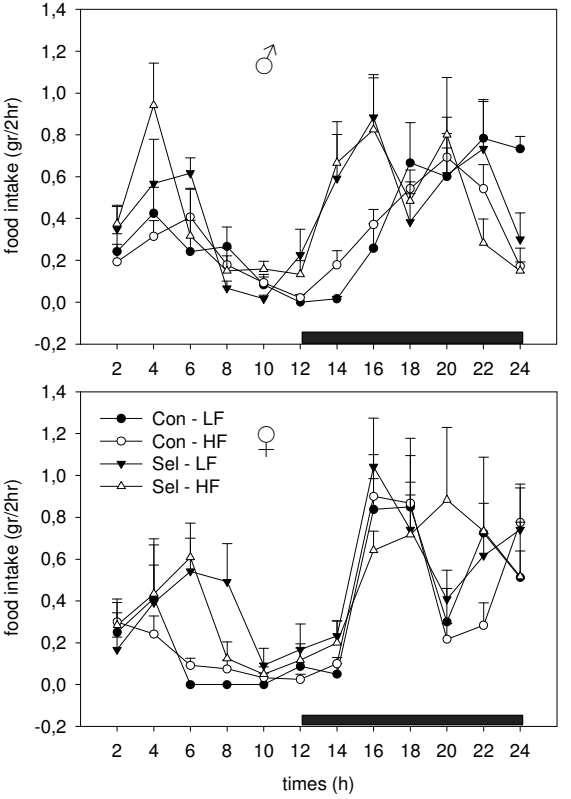

Figure 5. Food intake in control (Con) and selected (Sel) male (upper panel) and female (bottom panel) mice feeding a low-fat (LF) or high-fat (HF) diet. Dark phase is indicated by the black horizontal bar at the bottom of the graph.

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.

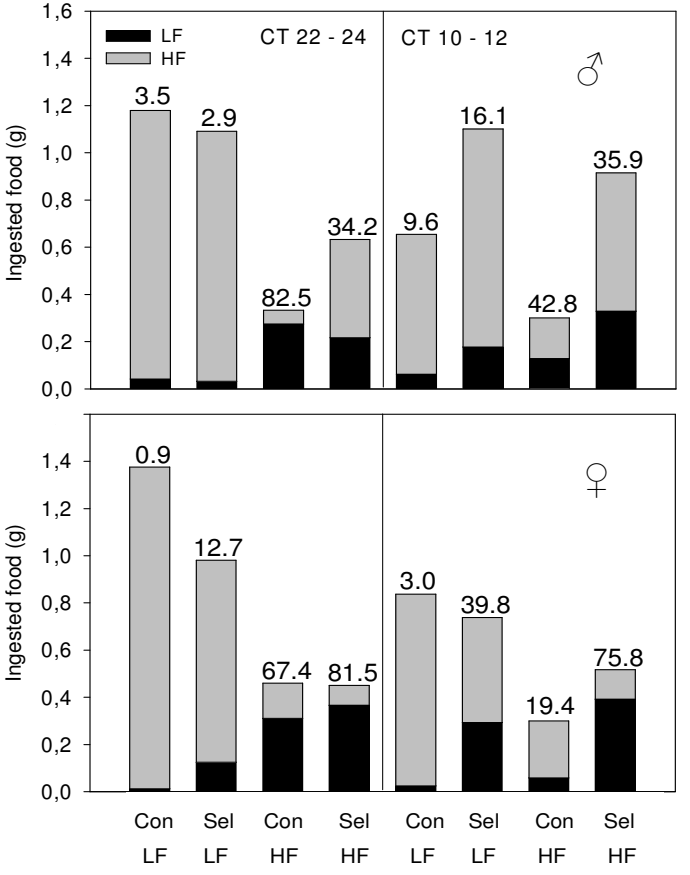

Figure 6. Food selection test in control (Con) and selected (Sel) male (upper panel) and female (bottom panel) mice at CT 22-24 (at the late dark phase, left panels) and at CT 10-12 (at the late light phase, right panels) either habituated to a low-fat (LF) or high-fat (HF) diet. Grey and dark fields 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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CHAPTER 4

EFFECT OF DIET ON THE ENERGETIC AND BEHAVIOURAL CHARACTERISTICS OF MOUSE LINES SELECTED FOR HIGH ACTIVITY

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Summary

Rodents subjected to forced physical activity are resistant to diet-induced obesity, but the procedure often involves emotional stress. For this reason, we studied interactions between voluntary activity and parameters related to energy balance and emotional stress in control mice, and in mice with a trait for increased wheel running behaviour. These latter animals show more voluntary behaviour even in the absence of running wheels. When subjected to a 40% high fat (HF) diet with added refined sugars, the activity selected lines were HF diet obesity resistant, with increased food intake, similar absorption rates and higher non-exercise thermogenesis (NEAT) relative to their randomly bred controls. While activity selected females had a higher anxiety level at baseline than control, this effect reversed on a HF diet, without concomitant changes in plasma levels of corticosterone. Since activity selected lines had a lower HF preference than controls in a two choice test of HF food vs. high-carbohydrate food, these data may be viewed such that the activity lines, and in particular the females improve their mood on a HF diet, without necessarily liking the HF diet more than control mice.

1. Introduction

In an attempt to unravel underpinnings of mammalian energy balance regulation and its derangements, numerous studies have been performed using rodents with traits for obesity proneness and resistance. Through experiments with these animals many candidate genes have been discovered involved in energy balance regulation (Brockmann and Bevova 2002). Selective breeding of rodents lines for diet-induced obesity refined these investigations by using diet as a default trigger, and allowed tracking the aetiology of obesity proneness-and resistance (Levin et al. 1997). It is currently believed that traits for obesity or resistance to it are not caused by single gene differences but is the result of multiple genes that interact at different levels. Indeed, in the seminal work of Singer and colleagues using chromosome substitution strains of mice, they identified that the trait for obesity involves genes almost on every chromosome. Secondly, they observed that high-fat diets differing in the level of refined sugars distort energy balance regulation probably via different mechanisms (Singer et al. 2004).

Another determinant of energy balance regulation is energy expenditure, but has been investigated not as extensively. In general, studies in rats and mice showed a decrease in body fat content in these animals when they had access to a running wheel even though their food intake increased (Tokuyama, Saito, and Okuda 1982; Looy and Eikelboom 1989; Bell, Spencer, and Sherriff 1997; Bell and McGill 1991). One approach to examine the effects of a high level of physical activity on energy balance regulation is to study this in animals that have been selectively bred for increased running wheel activity. Swallow et al consistently observed that selection lines ate more food compared to the controls (Swallow, Carter, and Garland, Jr. 1998b), but remained at a lower body weight during the entire period of the experiment compared to the controls. The activity-selected animals also showed lower fat accumulation which can explain the lower body weight (Swallow et al. 2001). A more recent study by Vaanholt et al showed that selected females, when fed a 60% high fat diet without refined sugars, had higher food intake than the controls but unlike the controls they showed remarkable resistance to weight gain on this high fat diet, even in the absence of running wheels. The activity-selected animals showed a higher mass corrected daily energy expenditure and had a higher resting metabolic rate compared to controls, and this was particularly evident in the females (Vaanholt et al. 2008). Thus, besides increased energy turn-over due to increases in spontaneous activity (Dauncey 1990), activity selected mice display increased metabolic rate independent of differences in activity levels. Together, these mechanisms contribute to resistance to high-fat diet-induced obesity.

As mentioned above, high fat diets differing in the level of refined sugars (i.e., sucrose) may cause distorted energy balance regulation via different mechanisms (Singer et al. 2004). For this reason, we aimed at reconciling the diet-obesity resistance of activity selected mice in the condition when these animals are feeding a high fat diet with an increased level of the refined sugar sucrose. Since it was shown in previous Chapters of this thesis and a number of published

reports (Girard et al. 2001; Swallow et al. 2001; Koteja et al. 1999; Gammie et al. 2003; Carter et al. 2000; Garland, Jr. et al. 1995; Vaanholt et al. 2007a; Belke and Garland, Jr. 2007; Malisch et al. 2009) that selective breeding for physical activity causes co-selection of several personality traits (including exploratory behavior, anxiety, stress sensitivity, and stereotypic behavior), we aimed at exploring these in control and activity selected mice in the face of feeding the high-fat high sucrose diet. The main hypothesis which we tested was that activity selected animals would be equally resistant to obesity on this high fat – high sugar diet as on the previously applied high fat alone diet, and that this obesity resistance would parallel outcomes of the behavioral tests.

2. Materials and Methods

2.1. Animals and housing

Thirty-six breeding pairs of Hsd:ICR mice (*Mus domesticus*) selected on high wheel-running activity (lines 7 and 8) for 50 generations and their random bred controls (lin 2) were obtained from T. Garland Jr, Riverside, CA (For a detailed description of the selection procedure see (Swallow, Carter, and Garland, Jr. 1998a). From these founder mice, separate breeding lines were continued at our facilities in Haren and further selected according to the same criteria. In the current experiments, 93 mice of the 52nd generation were used. From the age of 7 weeks onward, they were individually housed in standard cages (Macrolon Type II, UNO Roest Vast Staal B.V., Soest, NL) in the same room with an ambient temperature of 22±1°C. At the start of the experiment all animals had *ad libitum* food either a healthy fibered low-fat diet (LF) (3.8 kcal/g; 58 % carbohydrate, 6 % fat, 22 % protein; Standard lab chow RMH-B 2181, HopeFarms BV, Woerden, NL) or a 40 % fat diet, additionally containing sucrose (HFS) (4.7 kcal/g; 30 % carbohydrate, 45 % fat, 18 % protein; AB Animal Diets, Woerden, NL). and water; they were on a 12:12 light-dark cycle (lights on at 8:00). Wood shavings and EnviroDry® were used as bedding material.

2.2. Body mass and food intake

Four weeks before the start of the experiments, male and female mice of the different lines were assigned to cohorts either receiving a low-fat unrefined carbohydrate rich diet (LF), or a 40% high-fat diet with 25% added sucrose (HFS). Body mass and food intake of the LF and HFS feeding mice were measured weekly between 12:00 and 14:00 over the course of the entire diet manipulation. Intake was corrected for spillage.

2.3. Diet preference test

Diet preference of all animals was tested by allowing animals access to the LF and the HFS diet during a two-hour interval 1) in the evening at 20:00 at the beginning of their active phase or 2) at

8:00 in the morning at the beginning of their inactive phase. The active phase diet choice was done at the age of 12 weeks and the inactive phase diet choice was done at the age of 14 weeks. The food was provided in the lid of the cage, separated by the water bottle. Over the two-hour period, mice were allowed to eat from the food without the experimenter being in the room. Thereafter, the food was removed and intake was assessed, and animals were readjusted to their 'normal' diet.

2.4. Elevated plus maze

To determine the anxiety levels of mice, they were tested on an elevated plus maze over the period of week 11 to week 13. Plus maze tests separated from diet choice tests for more than two days. The maze consisted of two open arms and two closed arms facing each other, 40 cm above the floor. Each arm was 29 cm long and 5 cm wide. The closed arms had 15 cm high walls with a closed top. At the beginning of an experiment, the mice were placed at the center (5 x 5 cm) of the elevated plus maze after which the experimenter left the room. The animal was then videotaped during 5 minutes and the behavior on the plus maze was scored using Eline software. This way the time spent on each arm and the crossings from one arm to another was recorded. After finishing testing an animal the maze got cleaned with water and dried, before placing a new animal on the plus maze. The time spent on each of the different arms was analyzed, it represents the animal's anxiety level, the more time spent on the open arms the less anxious the mouse is and vice versa.

2.5. Stress test

At the age of 14-15 weeks a novel cage stress test was performed on all groups. At the start of the experiment, a blood sample was taken in their home cage by tail clip and 30-40µl of blood was collected in a heparinized tube. After 5 minutes, the animals were moved to a brightly lit room adjacent to their habituated room, and placed in a clean empty cage (identical to their home cage but without bedding). They were left in this cage for 15 minutes and then another blood sample was collected. After the experiment the blood was centrifuged and the blood plasma was collected and stored at -80°C. Later the amount of corticosterone in the plasma was measured by RIA (Linco Research, Nucli lab, The Netherlands).

2.6. Respirometry measurements

In week 21-23, animals were moved into respirometric chambers to determine oxygen consumption (VO_2, l/h) and carbon dioxide production (VCO_2, l/h) by indirect calorimetry for 24 hours. Eight animals could be measured simultaneously. Oxygen and carbon dioxide concentration of dried inlet and outlet air (drier: molecular sieve 3 Á, Merck) from each chamber was 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 was measured with a mass-flow controller (Type 5850 Brooks). Ambient temperature in the chamber and cage, as well as activity (with passive infra-red detectors) were measured simultaneously. Samples were collected every 10 minutes 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 (standard chow or fat) and water were provided *ad libitum*.

Heat production (HP, kJ/h) was calculated using the following equation: HP= (16.18 x VO_2) + (5.02 x VCO_2) (Romijn and Lokhorst 1964). Resting metabolic rate (RMR, kJ/h) was defined as the lowest value of heat production calculated as the running mean over half an hour and was calculated for the first and second day in the respirometer separately. Maximal heat production was also calculated as the running mean over half an hour. In addition, the average heat production (daily energy expenditure: DEE, kJ/d), respiratory quotient (RQ= VCO_2/VO_2) and PIR (passive infrared) activity were calculated for both consecutive days.

2.7. Bom calorimeter measurements

Directly after respirometry, feces of animals were collected from the saw-dust bedding and were weighed. Then the caloric content of the feces was measured by bom calorimeter. First a known amount of benzoic acid (known energy content of 6320 cal/g) was combusted in the bom calorimeter. This was done three times and the average of those three measurements was used as the reference point for the samples of the feces. Then samples of the feces were combusted and the heat production was compared to the references to determine the energy content of the samples. With these data combined with the assessed food intake during the 24 hours in the respirometer, this enabled us to estimate metabolizable energy intake (MEI).

2.8. Data analysis

All of the data were analyzed using Statistica 7. The body mass and food intake data that were measured during 12 weeks were analyzed with repeated measures ANOVA. For the analysis of the other data general linear models were applied. To test the effect of diet within each line, the data from each line was analyzed separately with a general linear model. To test all subgroups a contrast test was performed.

3. Results

3.1. Body mass and food intake

Figure 1 shows changes in body weight of the mice over time when they were feeding a HFS or LF diet. Mice from the selection lines have a significantly lower body mass compared to the control animals (♂: $F(2,44)=25.53$; $p<0.001$ and ♀: $F(2,40)=39.95$; $p<0.001$). This is in agreement with the other studies that were done with the activity selected lines compared to the controls (Swallow et al. 2001; Vaanholt et al. 2008; Vaanholt et al. 2007b). Body weight gain over time was higher in line 2 ($F(11,44)=8.16$; $p<0.001$) and line 7 ($F(11,44)=2.69$; $p<0.001$) males on the HFS diet than on the LF diet. This pattern is not seen in the females.

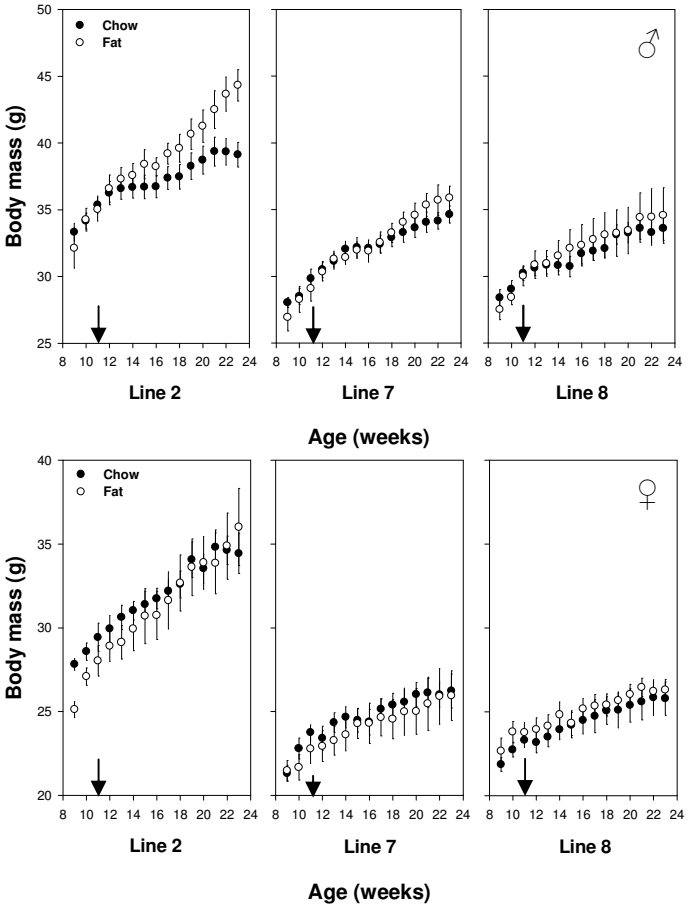

Figure 1. The average body mass over time for all groups in male (top panels) and female mice (bottom panels). The arrows depicts the age at which the HFS diet was provided.

The average daily food intake (per week) of the mice is shown in figure 2. In males food intake of line 8 ($p=0.004$), but not line 7 mice was generally increased above the levels found in line 2. In females, both line 7 and line 8 showed increased food intake above the levels observed in line 2 females ($F(2,40)=19.31$; $p<0.001$). Line 2 (♂: $F(1,44)=26.40$; $p<0.001$ and ♀: $F(1,40)=50.32$; $p<0.001$) and line 7 (♂: $F(1,44)=33.10$; $p<0.001$ and ♀: $F(1,40)=5.12$; $p<0.047$) males and females showed clearly reduced food intake on the HFS diet compared to respective groups on the LF diet, except for line 8.

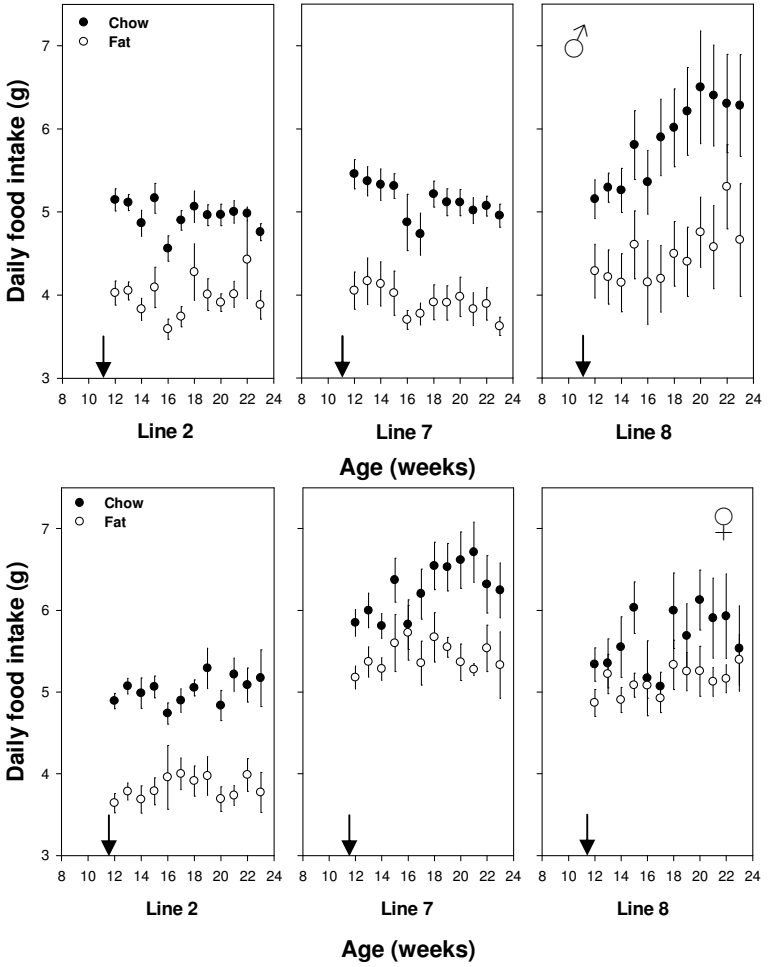

Figure 2. The average daily food intake over time for all the different groups in male (top panels) and female (bottom panels) mice. The arrows depicts the age at which the HFS diet was provided.

3.2. Absorption and growth efficiency

During the 24 hours in the respirometer, the amount of food eaten was assessed and the feces that were excreted were collected. The total calories of the food intake and the feces was determined (males only), and with this data the percentage of absorption of the food was calculated (figure 3). The high absorption rates of about 80-90% are comparable to data collected in other studies done with mice or rats (Carvajal et al. 2000; Chen et al. 2003; Lo et al. 2008; Santos, Coelho, and Coelho 2008). The animals that were on HFS diet show a significantly higher percentage of absorption compared to the chow fed animals ($F(1,37)=14.82$; $p<0.001$) irrespective of line.

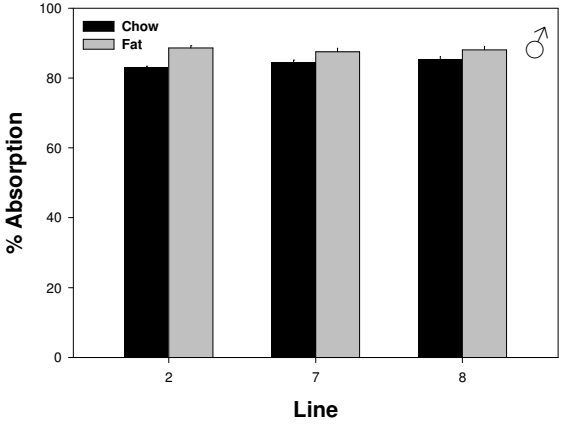

Figure 3. The percentage of food that was absorbed from the total food that the male mice consumed during their time in the respirometer.

Growth efficiency was calculated by dividing the weekly food intake by the weekly change in body mass. The HFS-fed animals had a significantly higher growth efficiency than the LF-fed animals (♂: $F(1,44)=26.30$; $p<0.001$ and ♀: $F(1,40)=11.12$; $p=0.002$). Line 2 males on the LF diet had a significantly higher growth efficiency compared to line 8 ($F(2,44)=4.64$; $p=0.025$) and for the females on the HFS diet the growth efficiency of line 2 was significantly higher than that of line 7 and 8 females ($F(2,40)=4.98$; $p=0.012$).

3.3. Respirometer measurements

From the data output of the respirometer the daily energy expenditure (DEE) could be determined, which is shown in figure 5. Overall females had a higher DEE when feeding the

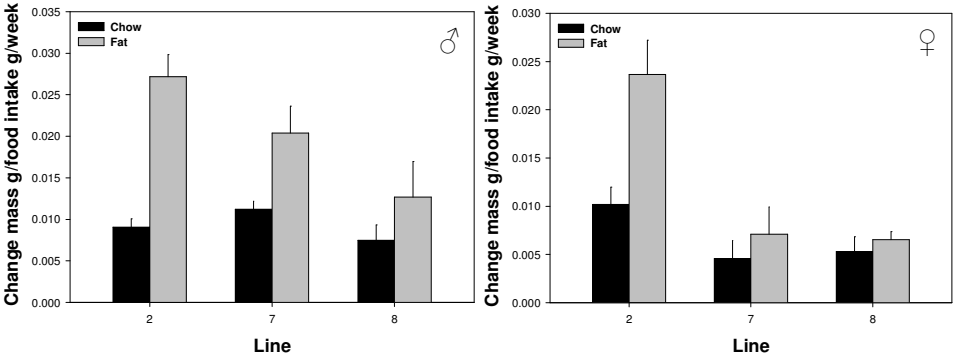

Figure 4. The average growth efficiency over a period of 12 weeks in male (left panel) and female (right panel) mice.

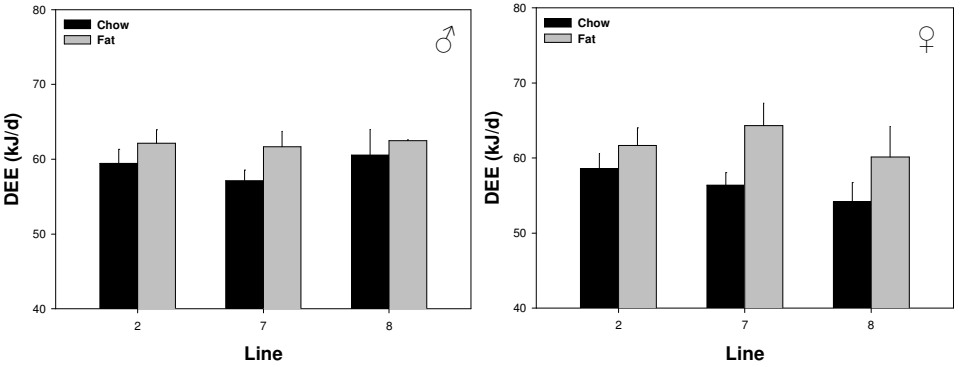

Figure 5. The daily energy expenditure (DEE) measured over 24 hours in the respirometer in male (left panel) and female (right panel) mice.

HFS diet than when they were feeding a LF diet ($F(2,39)=6.35$; $p=0.016$). In males a similar pattern can be seen but the difference was not significant.

Resting metabolic rate (RMR) is significantly influenced by body mass. Therefore, RMR is depicted as the lean mass-specific RMR in figure 6. The males show a significant line*diet interaction ($F(2,44)=3.52$; $p=0.040$) and line 2 animals appeared to have slightly lower RMR on the HFS diet compared to when they were feeding a LF diet. Lines 7 and 8 males, on the other hand, showed a slightly higher RMR when feeding the HFS diet (table 6). None of the groups, however, showed a significantly higher or lower RMR compared to the other groups in the post-hoc analysis, but there appears to be a trend of a higher RMR in the selection animals.

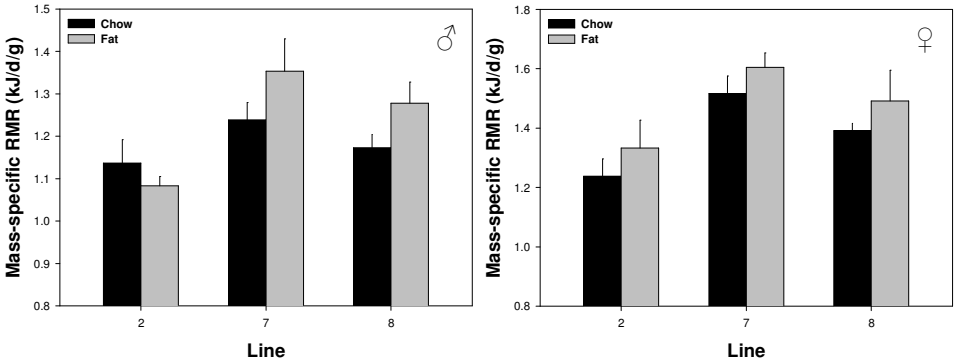

Figure 6. The resting metabolic rate calculated from the measurements in the respirometer in male (left panel) and female (right panel) mice.

By subtracting RMR (i.e., non corrected for body mass) from DEE in each animal, this reveals energy allotted to thermogenesis by spontaneous activity and diet; the so-called non-exercise activity thermogenesis (NEAT), and the averages of energy expenditure by RMR and NEAT are depicted in figure 7. The effects of line and diet on the RMR have already been discussed in the previous section. Line 8 males have a significantly higher NEAT compared to the line 2 males ($F(2,44)=4.36$; $p=0.020$), irrespective of diet. In the females, both line 7 and 8 show a significantly higher NEAT than the control line ($F(2,44)=3.65$; $p=0.036$), irrespective of diet.

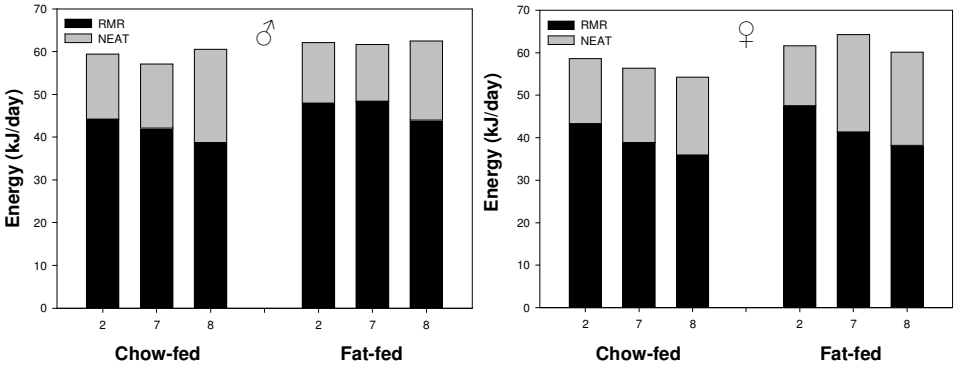

Figure 7. Energy spent during the time in the respirometer, consisting of the RMR and NEAT in male (left panel) and female (right panel) mice.

3.4. Diet choice

The results of the diet choice test are presented in figure 8. The two diet groups had markedly different effects on diet selection; i.e., mice feeding LF-fed diet as their regular food almost exclusively ate HFS-diet during when given the choice between HFS and LF food, at both the inactive and active phase. Mice feeding the HFS diet as their regular food ate both LF as well as HFS diet during the choice test. This pattern is most clearly seen for the test in the morning, when all lines of HFS-fed animals ate considerable amount of LF diet (♂: $F(1,43)=47.23$; $p<0.001$ and ♀: $F(1,40)=12.69$; $p<0.001$). In the males, lines 7 and 8 eat significantly more LF diet than the line 2 males during the inactive morning phase ($F(2,43)=6.10$; $p=0.005$). In the females, only line 8 mice select more LF diet compared to line 2 mice ($F(2,40)=5.66$; $p=0.035$), and this was predominantly seen in the active phase (see table 6).

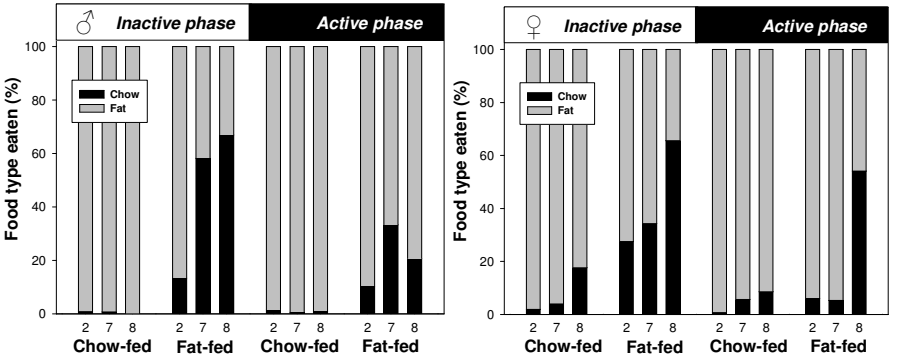

Figure 8. Diet choice test showing the food preference for both the inactive and active phase in male (left panel) and female (right panel) mice. Total intake is set at 100% for each animal, and the subsequent intake from HFS and LF diets are presented as grey and black bars.

3.5. Elevated plus maze

The results of the elevated plus maze test are shown in figure 9. Diet did not affect anxiety levels (expressed by time in open versus closed arm) in males. The females on the other hand, showed a significant line*diet interaction ($F(2,40)=5.76$; $p=0.007$). The three lines all showed a different response to the test. The LF-fed line 2 females spent significant less time in the closed arms $F(2,40)=6.45$; $p=0.004$) (indicating reduced levels of anxiety relative to selected lines), and line 8 females on the LF diet showed the lowest presence in the open arms ($F(2,40)=5.76$; $p=0.007$) (with line 7 as the intermediate). On the HFS diet, however, this picture in the females completely reversed. In males feeding the LF diet, a pattern with some similarity was found with the females on the LF diet, but these effects did not attain significance. On the HFS diet, males of the different showed exactly similar responses on the plus maze.

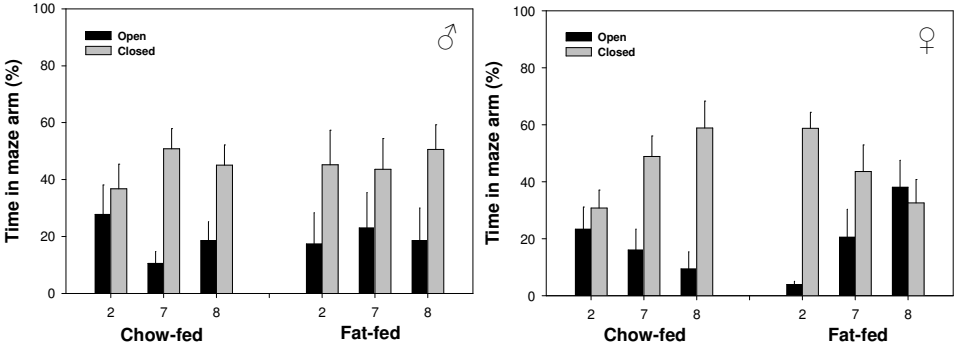

Figure 9. Elevated plus maze test showing the percentage of time spend on both the open arm and the closed arm in male (left panel) and female (right panel) mice.

3.6. Stress test

The results of the novel cage test on plasma corticosterone levels are shown in figure 10. At baseline, no differences were observed plasma corticosterone levels in males and females between lines and diets. During the stress, however, male mice showed a line effect ($F(2,41)=3.55$; $p=0.038$), and post-hoc analysis revealed that line 7 had lower plasma corticosterone levels than line 2 males irrespective of diet. Effects in the females pointed in the opposite direction (i.e., with plasma corticosterone levels higher in line 7 mice compared to line 2 mice), but this effect failed to reach significance.

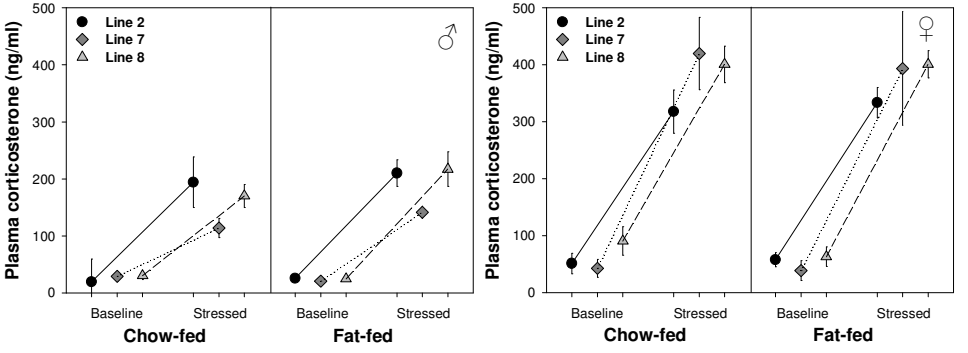

Figure 10. Stress test showing the amount of corticosterone measured in plasma for the baseline levels measured before the test and the stress levels measured after the test.

4. Discussion

The present study investigated the effects of feeding a diet consisting of 40% saturated fat and 25% refined sugars on energy balance regulation and an array of behaviors in control mice as well as in mice selectively bred for increased wheel running behavior. It was hypothesized that the high activity lines, and in particular the females would be resistant to the obesogenic actions of the HFS diet, and the control line would be obesity prone. Since selection for wheel running behavior has been shown to cause co-selection of various traits (i.e., which may be helpful to sustain endurance behavior), these co-adaptations may also be hypothesized to be resistant to change.

Consistent with our hypothesis and previous observations was the finding in the present study that the line 7 and line 8 activity-selected males and female mice did not respond with weight gain on the HFS diet compared to those feeding the LF diet. Mechanisms underlying this effect included increased mass specific-resting metabolic rate (RMR) and a higher level of non-exercise activity thermogenesis (NEAT) (Donahoo, Levine, and Melanson 2004). Although not assessed in the present study, the latter effect is obviously associated with increased levels of spontaneous activity in the activity selected animals which has been shown to be displayed by these animals even without running wheels (Rhodes et al. 2001). The effects on RMR were significantly increased in activity selected males compared to the control males (and with a strong trend in the females), but depended on diet. Thus, while RMR decreased slightly in the control males in the HFS condition relative to the LF condition, RMR increased in line 7 and line 8 males in the HFS condition. In contrast, the level of NEAT was significantly increased in the activity selected mice irrespective of diet in males, but in females this effect was strongest in the HFS condition. Together, this rendered the highly active mice less growth efficient than the control mice. Differences in food absorption (at least in the males) could not account for this effect since we did not find differences in the energy content of feces by bom calorimetry among lines.

The diet resistance in the line 7 and line 8 females is remarkable in light of the findings that they ingested between 15~20% more food than the control females did, and hardly responded with a reduction in food intake when subjected to the HFS diet. The latter would be a "normal" response when animals are subjected to high amounts of dietary fat (van Heek et al. 1997). Opposite to our previous observation was the finding in the present study that not the control females, but the control males were prone to weight gain on the HFS diet. We have no data on the body composition analysis, but since diet manipulations were started at 11 weeks of age, it is unlikely that the effects are not attributed to differences in adiposity. Furthermore, we do not know whether the increased weight gain is due to the differences in dietary fat percentage (i.e., 60% fat in our previous studies vs. 40% fat in the present) or the addition of the sucrose in the present study. Harris et al (Harris, Bowen, and Mitchell 2003) demonstrated previously that female, but not male mice retain leptin sensitivity on a 45% HF diet. Since blunting of leptin signalling is a major cause underlying weight gain in rodents (Ruffin et al. 2004), and might

underlie differences in resistance and proneness to diet-induced obesity (Levin and Dunn-Meynell 2002), this might very well explain differences in weight gain in the control and activity selected animals in the present study.

Despite resistance to weight gain in the selectively bred females, striking line differences were obtained in the female mice subjected to the elevated plus maze test. When feeding the LF diet, the line 2 females were the least anxious (indicated by the highest percentage of time present on the open arms) on the plus maze and the line 8 were most anxious. Line 7 LF feeding females showed an intermediate response. This confirms the findings of Chapter 2. On the HF diet, however, this pattern completely reversed, now the line 2 females were most anxious and line 8 ones the least. Thus, in control females, feeding the HFS diet increased anxiety levels whereas it reduced it in activity selected ones. Differential effects of dietary fat and sugars have been observed before on stress sensitivity and anxiety levels (Torres and Nowson 2007), and the direction of change may depend on the previous experience of animals (van Dijk and Buwalda 2008). One idea might be that the HFS diet enhances mood (Dallman et al. 2003) particularly in the highly activity females mice when they are not allowed to run in wheels, and through this mechanism could dampen anxiety and/or could replace diminished reward from abstinence of wheel running. In control females on the other hand, the HFS diet may have adverse effects on its own, which would be consistent with the study of Souza et al. (Souza et al. 2007).

If a diet is mood-enhancing, it would be of interest to test whether they also select more of it when given a choice of diets (la Fleur et al. 2007). We observed that the high-activity mice given a choice between the LF and HFS diet generally selected more of the LF diet than the line 2 mice did. The relative higher LF diet preference in the highly active mice was evident in males, and in females only in line 8. This differs slightly with the findings in Chapter 3, where particularly the line 7 females selected more the LF diet (although line 8 was not tested under those circumstances). Thus, if the HFS diet lightens up line 7 and line 8 animals (i.e., resulting in less anxiety in the plus maze test), this apparently does not correspond with the direct appreciation of the diet. While it is difficult to dissociate "wanting" and "liking" effects (Berridge, Robinson, and Aldridge 2009) of the diets in a two-hour diet choice test, one might speculate that the HFS diet gives less immediate reward in the high activity mice than the LF diet, but may enhance mood and dampens anxiety on the long-term. Another reason for selecting a certain diet could be that its macronutrient content is metabolically more appropriate (see Chapter 3). The present study does not confirm nor reject the latter possibility.

The differences in anxiety behavior and food selection were not reflected by differences in plasma corticosterone responses in the novel cage test in the present study. Line 7 males appeared to have significantly lower levels of plasma corticosterone than line 2 males irrespective of diet, but no differences were observed in the females between lines and diets. Thus, although Girard et al. found evidence for suppressed stress sensitivity in the selection mice compared to the controls (Malisch et al. 2008; Girard and Garland, Jr. 2002) - an effect which would be in line

with reports showing stress relief as a result of physical activity (Salmon 2001) - we did not unequivocally confirm these ideas in the present study.

In summary, activity selected mice were resistant to weight gain when chronically subjected to a HFS diet, and changes in RMR and NEAT contributed to this effect. Male control mice, on the other hand, increased body weight on this HFS diet. The resistance to weight gain in the activity selected mice did not parallel resistance to the effects of the HFS diet on affective traits. On the contrary, the levels of anxiety and appreciation of the diet were different between control and selected mice in the LF condition, but moreover differentially regulated in the HFS diet condition. Furthermore, if animals in the present study would have been housed in cages with access to running wheels, it is quite possible that anxiety levels in the LF diet condition would have been lower in the activity selected animals (Salmon 2001). In the present study, the highly active mice seem to recover by feeding a HFS instead, without necessarily appreciating this diet more than control mice do. As such, physical activity and nutrition may be regarded as default systems that determine mental and physical health.

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CHAPTER 5

MICE SELECTIVELY BRED FOR HIGH WHEEL RUNNING ACTIVITY HAVE INCREASED GROWTH EFFICIENCY DURING LACTATION

I. Jónás, K. A. Schubert, T. Garland Jr, A. J.W. Scheurink, C. Nyakas, and G. van Dijk

Summary

We hypothesized that high voluntary physical activity would negatively affect growth efficiency (GE) during lactation in mice. We assessed mother and litter characteristics during lactation under low fat (LF) and high fat (HF) dietary feeding conditions in mice selectively bred for high wheel-running behavior (lab-designated lines 7 and 8) and in a non-selected control line (line 2). At birth, litter weights were lower in HF feeding mothers irrespective of line, and litter sizes were larger in line 7 and 8 relative to line 2, irrespective of diet. Over the course of lactation, all females lost pups, but HF feeding line 7 females lost significantly more than others. At peak lactation (post-gestational days 13-16), litter weights but not maternal body masses of HF feeding mothers became increased above LF ones. GE (calculated as weight gain of mothers and litters divided by absorbed energy of mothers) during peak-lactation was higher in HF feeding than LF feeding mothers, and statistically interacted with line, with both line 7 and 8 higher than line 2 in the HF diet condition. Regression analysis revealed that this HF-diet effect to increase GE was particularly the case at large litter sizes. It is concluded that a trait for increased physical activity augments GE during HF diet feeding, but may have disadvantageous effects on pup survival.

1. Introduction

Lactation is an energetically demanding occupation for mammals. In human beings, the incremental energy cost of lactation adds approximately 25% to the normal energy requirements, and this is met by an increase in food intake, mobilization of body fat reserves, and an increase in metabolic efficiency (reviewed by (Butte and King 2005). In rodents, the energy cost of lactation is estimated to increase even four-fold above normal energy requirements (Johnson, Thomson, and Speakman 2001a). The maximum energy consumption and expenditure - termed sustainable energy intake (SusEI) and sustainable metabolic rate (SusMR), respectively - during this period have been suggested to be limited intrinsically by aspects of physiology, and this concept has been outlined in a number of mouse studies by Krol and Speakman (Krol and Speakman 2003; Speakman and Krol 2005; Krol, Murphy, and Speakman 2007) and earlier workers.

Over the course of lactation, laboratory house mice first show a linear increase in energy intake and then it stabilizes to a maximum intake between days 13 and 16. This period has been termed "peak lactation" (Thompson and Nicoll 1986). At peak lactation, Johnson et al observed that the amount of ingested food correlates positively with litter size as well as litter mass ((Johnson, Thomson, and Speakman 2001a). Furthermore, resting metabolic rate (RMR) and daily energy expenditure (DEE) of lactating dams also increase with increasing litter size (Johnson, Thomson, and Speakman 2001a; Johnson, Thomson, and Speakman 2001b). Thus, litter size may be important to determine the mother's energy procurement to her pups, as variation in litter mass was observed to be linearly related with litter size, without affecting individual pup mass (Johnson, Thomson, and Speakman 2001a). However, when litter size increases above a certain number, SusEI becomes limited, which subsequently causes a reduction in average pup mass. One hypothesis by which SusEI is maximized states that a lactating animal has the risk of lethal overheating when it is feeding litters that are too large (Krol and Speakman 2003). Thus, the maximum capacity to dissipate heat would then limit individual pup growth above a certain litter size.

In the current study, the relation between maternal body weight, SusEI and resting metabolic rate, litter size, litter weight, and individual pup growth was investigated during lactation of female mice that were selectively bred for high wheel-running activity and in non-selected controls. Female mice from the breeding line for increased running wheel activity have low body masses together with a high level of energy turn-over (Vaanholt et al. 2008). On a high-fat/high-sucrose "Western"-type diet they are markedly hyperphagic, but resistant to becoming obese due to increased spontaneous activity and elevated heat production. In contrast, control mice on this diet suppress their food intake, but have dramatically increased growth efficiency (van Dijk et al. in preparation), and thus become obese (Vaanholt et al. 2008).

The major aim of the present study was to investigate whether the increased level of performance (see also (Meek et al. 2009))of highly active mice alters afore-mentioned relationships between SusEI and litter characteristics during lactation when feeding a standard low-fat laboratory (LF) diet or a high fat/high sucrose (HF) diet. The one study reporting on

reproductive success and litter characteristics of these highly-active female mice from an earlier generation showed that they do not differ in reproductive output, litter size, and litter mass from control lines (Girard et al. 2002). When body masses of lactating females were taken into account, selected mice tended to wean relative heavier litters than control females. Therefore, it may be predicted that energy procurement to offspring of highly-active mice would be higher and/or more efficient, particularly on a HF diet. Alternatively, it may be predicted that individual pup growth becomes limited by activity of muscular tissue of highly-active mice feeding a HF diet, which drains energy away from milk production, thus leading to a trade-off. This has been mentioned by Krol et al. as a "peripheral" limiting factor to offspring development (Krol, Johnson, and Speakman 2003). The likelihood of such a trade-off is supported by the observation that, in a previous study, males from one of the selected lines did not exhibit an increased maximum workload when mice were forced to work (via wheel running) for food (Vaanholt et al. 2007).

2. Materials and methods

2.1. Animals and housing

Mice used in this study were selectively bred for voluntary wheel-running behavior (the base population was the Hsd:ICR strain) over 49 generations and were obtained from T. Garland Jr, Riverside, CA. In the original selection protocol, eight lines of mice were created (4 selected and 4 control) (Swallow, Carter, and Garland, Jr. 1998). Breeding lines were maintained at our facilities in Haren without further selection for wheel-running activity. Mice were typically housed in standard cages (Macrolon Type II, UNO Roestvaststaal BV, Zevenaar, NL) in the same room with *ad libitum* access to water and a low-fat (LF) standard laboratory mouse chow (3.8 kcal/g; 58 % carbohydrate, 6 % fat, 22 % protein; RMH-B 2181, HopeFarms BV, Woerden, NL) at an ambient temperature of 22±1 °C, and maintained on a 12:12 light-dark cycle with lights on at 8 am. Pine shavings and EnviroDry® were used as bedding material. 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.

From two selection lines (lab designation line 7, n=26; and line 8, n=26) and one control line (line 2, n=24), virgin female mice at the age of 4.5 months were paired with males of equal line. Two weeks before pairing, about half of the mice from each selection/control group were switched from the LF diet to a high-fat (HF) diet, additionally containing sucrose (4.7 kcal/g; 30 % carbohydrate, 45 % fat, 18 % protein; AB Animal Diets, Woerden, NL). The others of each group were left on the LF diet. After three weeks of pairing, males were removed and females were left to deliver and raise their litters until weaning (see Table 1).

Table 1. The number of paired females per line either feeding a low-fat (LF) or high-fat (HF) diet, successful deliveries/weanings and the total pup counts at these time points.

Line	2 Control		7 Selected		8 Selected	
Diet	**LF**	**HF**	**LF**	**HF**	**LF**	**HF**
Females (n)						
paired	13	11	12	14	12	14
successful deliveries	13	9	10	13	11	11
successfully weaned nests	13	9	9	10	10	11
Pups alive (n)						
at birth	117	65	116	123	116	121
at weaning	114	62	77	73	95	119

2.2. Mother and offspring characteristics from parturition to weaning

Maternal body mass was assessed once prior to breeding and after parturition, and daily throughout the period of lactation. Food intake was also measured daily throughout lactation. Bedding was also daily checked for spilled or crumbled food (Koteja et al. 2003). At peak lactation (between day 13-16), besides assessing food intake, feces produced by the female and her offspring over these 3 days were collected. The energy content in dried, homogenized feces and food was determined using a bomb calorimeter (CBB 330, standard benzoic acid 6320 cal/g, BCS-CRM No.90N). After day 16 (before offspring started to eat from food hoppers), mice feeding the HF diet were switched back to feeding the LF diet.

The absorbed energy of the mothers at peak lactation was calculated from the difference between energy intake and energy content of the feces, and subsequently used to calculate efficiency of body weight gain of mother and pups (i.e., expressed as mg body weight gain/kJ food absorbed). On the last day of peak lactation (day 16), animals were subjected to indirect calorimetry to assess resting metabolic rate (RMR, kJ h^{-1}) (Johnson, Thomson, and Speakman 2001b). Each animal was placed in a 2 L Plexiglas metabolic chamber at thermoneutrality (30±1°C) for 4 hours. Home cage bedding was provided to reduce stress of novelty. During this period, females were separated from their litters (which remained in their home cage) and did not have access to food. Specifically, measurements included O_2 consumption (VO_2, ml h^{-1}) and CO_2 production (VCO_2, ml h^{-1}) with an open air flow system (Oklejewicz et al. 1997) with inlet airflow set at 20 l hr^{-1} (Brooks Type 5850 mass flow controller, Rijswijk, NL). Channels were sampled in sequence, with each measurement lasting one minute, and a reference channel measured at least once every five minutes. Inlet and outlet air were dried (3 Å molecular sieve drying beads, Merck, Darmstadt, Germany) and analyzed for gas concentrations with a paramagnetic oxygen analyzer (Servomex Xentra 4100, Crowborough, UK) and infrared gas analyzer for CO_2 (Servomex 1440). The system recorded the differentials in oxygen and carbon dioxide between dried reference air and dried air from the metabolic cages. Oxygen consumption was calculated using equation 2 of

Hill (Hill 1972) and expressed at standard temperature and pressure. We calculated the respiratory quotient (RQ) as VCO_2/VO_2. Heat production (HP, kJ h^{-1}) was calculated from the equation formulated by (Romijn and Lokhorst 1961):

$$HP = 16.18 * VO_2 + 5.02 * VCO_2$$

We defined the RMR at peak lactation as the lowest value of a 20-minute running mean of HP, typically the average of three consecutive measurement points which corresponded when the animals were fully resting, as detected with Passive InfraRed sensors (PIRs). Metabolic rate tended to decrease over time and reach a stable low level. Animals usually reached RMR after 3 hours in the measurement chamber. Following parturition, the number of pups and the total mass of the litters were assessed and mean pup mass was calculated. Measurements were done at birth (day 0-1), 13, 16 and 21 (weaning).

2.3. Statistical analysis

GLM Repeated Measures was used to analyze differences in body mass and food intake of females through lactation. GLM Univariate Analysis was used to analyze differences in energetic measures taken at peak lactation (food intake, RMR, PIR, energy content of feces, calculated absorbed energy and growth efficiency) and for differences in litter characteristics. Multiple regression analysis was performed to assess correlations between litter characteristics, as well as between litter size and mother characteristics at peak lactation to reveal line and diet effects. A value of $p<0.05$ (for 2-tailed tests) was considered significant for all tests.

3. Results

3.1. Mother characteristics

Before pregnancy, body weight of line 2 females was 22 % higher than that of line 7 and line 8 females ($F(2,55)=24.2$; $p<0.001$), irrespective of diet (Fig. 1). On the last day of pregnancy, a doubling was observed in body weights of females relative to the day of conception ($F(2,55)=15.01$; $p<0.001$), without any significant difference in body weight gain among lines or between diets. After delivery, body weight of line 2 females was approximately 15 % higher compared to line 7 and 8 females ($F(2,56)=15.09$; $p<0.001$), irrespective of diet. Within-subject repeated measures analysis over days 1 through 16 revealed that body weight of line 8 females increased significantly more (5.2±0.5 g) than that of line 2 females (1.4±0.8 g), while that of line 7 females gained intermediate weight (3.4±0.5 g) (line*time interaction: $F(30,840)=2.96$; $p<0.001$). Furthermore, as can be seen in Figure 1, females fed the HF diet gained significantly less weight, irrespective of line, than those fed the LF diet (diet*time interaction: $F(15,840)=1.73$; $p<0.05$).

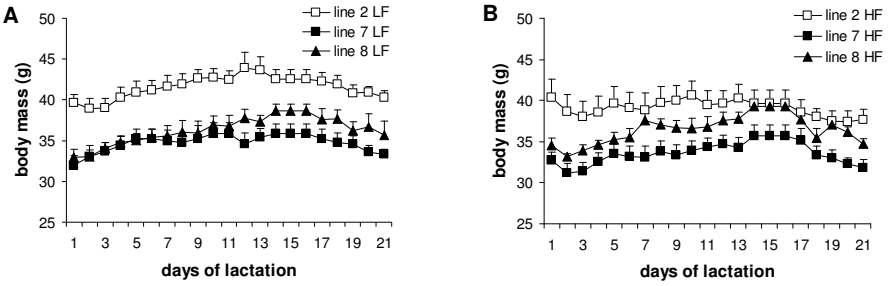

Figure 1. Body weight of line 2 (control line), line 7, and line 8 (selected lines) mothers fed a low-fat (LF, panel A) or a high-fat (HF, panel B) diet during lactation.

Within-subject repeated measures analysis over days 1 through 16 revealed that food intake (Fig. 2) of lactating females was increasing over time ($F(14,770)=46.18$; $p<0.001$). This effect interacted with line (line*time: $F(28,770)=2.01$; $p<0.01$), but not with diet. Specifically, line 2 mothers ate 3 times more over the 16-day period, whereas line 7 and line 8 mothers ate only 2.3 times more over the period of lactation. When GLM Univariate Analysis was performed for each day, line 8 mothers were found to eat the most on the first and second day after delivery in the HF diet group only (day 2: $F(2,60)=3.55$; $p<0.05$). Post-hoc differences were lost after day 2.

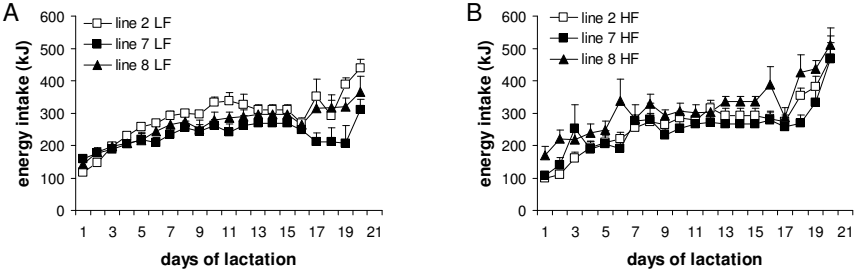

Figure 2. Energy intake of line 2 (control line), line 7, and line 8 (selected lines) mothers fed a low-fat (LF, panel A) or a high-fat (HF, panel B) diet during lactation.

3.2. Litter characteristics

At birth, litter sizes of line 7 and line 8 females were significantly larger ($F(2,61)=7.24$; $p<0.01$) than those of line 2 (see Table 2). Over the course of lactation, pups were found missing in all lines, but, on average, line 7 females lost significantly more pups per litter than line 2 and 8 females, irrespective of diet ($F(2,61)=8.68$; $p<0.001$). Litter weight at birth was lower in females fed the HF diet ($F(1,60)=4.73$; $p<0.05$), and litter weights of line 2 and 7 females contributed mostly to this effect. At day 13, however, litter weight of HF-fed mothers became higher

($F(1,56)=4.36$; $p<0.05$) compared to litters of LF-fed mothers. This effect was particularly observed in line 8, which persisted until day 16 ($F(1,56)=6.65$; $p<0.05$). At day 13, litter weight was also influenced by line ($F(2,56)=5.20$; $p<0.01$); i.e., litter weight of line 7 was smaller than litter weight of line 2 and line 8 females, which remained at day 16 ($F(2,55)=5.45$; $p<0.01$) and day 21 ($F(2,55)=7.88$; $p<0.001$). On day 21, effects of diet were lost, an effect presumably caused by the fact that mothers were switched back to the LF diet (see table 2).

Mean individual pup mass at birth was 14% lower in line 7 and 12% lower in line 8 than that of line 2 ($F(2,60)=14.87$; $p<0.001$), irrespective of diet. From day 13 onwards, these differences became larger; mean pup mass of line 7 was 29 % lower and line 8 was 27 % lower than that of line 2 ($F(2,54)=29.48$; $p<0.001$). Furthermore, mean pup mass became increased by diet irrespective of line from day 13 onwards ($F(1,56)=23.92$; $p<0.001$) (see table 2).

Table 2. Litter characteristics of line 2 (control line), line 7, and line 8 (selected lines) mothers fed a low-fat (LF) or a high-fat (HF) diet.

	2 Control		7 Selected		8 Selected	
	LF	**HF**	**LF**	**HF**	**LF**	**HF**
Litter size						
day 1	9.0 ± 0.8	7.2 ± 1.0	11.6 ± 0.5 #	9.5 ± 0.8	10.5 ± 0.6	11 ± 0.8
day 13	8.8 ± 0.8	6.9 ± 1.0	8.1 ± 1.3	5.8 ± 1.3	8.6 ± 1.3	10.9 ± 0.8
day 16	8.8 ± 0.8	6.9 ± 1.0	7.8 ± 1.3	5.6 ± 1.3	8.6 ± 1.3	10.8 ± 0.7
day 21	8.8 ± 0.8	6.9 ± 1.0	7.7 ± 1.3	5.6 ± 1.3	8.6 ± 1.3	10.8 ± 0.7
Litter weight						
day 1	16.1 ± 1.2	12.5 ± 1.6	17.8 ± 0.7	14.3 ± 1.2*	16.4 ± 0.7	17.4 ± 1.0
day 13	68.4 ± 3.8	71.4 ± 7.1	48.2 ± 5.9 ##	53.8 ± 8.1	54.4 ± 5.2 #	76.8 ± 6.1*
day 16	75.7 ± 4.4	82.5 ± 8.1	53.1 ± 6.3 ##	61.5 ± 10.0	62.3 ± 5.2	89.9 ± 6.2**
day 21	103 ± 6.4	104.8 ± 10.6	65.6 ± 7.9 ##	74.3 ± 12.4	83.8 ± 8.3	113.6 ± 8.5
Mean pup mass						
day 1	1.8 ± 0.1	1.8 ± 0.0	1.5 ± 0.0 ##	1.5 ± 0.0	1.6 ± 0.0 ##	1.6 ± 0.1
day 13	8.3 ± 0.5	10.9 ± 0.5**	5.5 ± 0.4 ##	7.2 ± 0.5*	5.9 ± 0.3 ##	7.1 ± 0.4*
day 16	9.1 ± 0.5	12.6 ± 0.7**	6.3 ± 0.4 ##	8.8 ± 0.5**	6.9 ± 0.4 ##	8.4 ± 0.5*
day 21	12.3 ± 0.6	16 ± 0.8**	7.9 ± 0.5 ##	10.4 ± 0.8*	9.4 ± 0.6 ##	10.6 ± 0.6

# denotes significant difference with line 2 (line effect) (#, $p<0.05$; ##, $p<0.01$). *denotes significance difference with LF diet (diet effect) (*, $p<0.05$; **, $p<0.01$).

3.3. Peak lactation

3.3.1. Maternal energetic characteristics

Table 3 shows maternal energetics during peak lactation (day 13-16). The total weight of food eaten during peak lactation was significantly influenced by diet ($F(1,59)=12.93$; $p<0.001$). Specifically, HF-fed mothers ate 18.2 % less than LF-fed females, without any effects of line. Conversion to energy content of the food revealed that energy intake (in kJ) was not significantly

altered by diet or line. Resting metabolic rate (RMR assessed on day 16 of lactation) was significantly lower in females fed the HF diet ($F(1,52)=19.18$; $p<0.001$) than that in females fed the LF diet. No line or interaction effect between line and diet was observed. The activity of lactating females (measured by PIR detection) was affected by line ($F(2,52)=5.98$; $p<0.01$) with line 7 being more active than line 2, and line 8 being intermediate. The total dry weight of feces during peak lactation was 42.7 % lower in females on the HF diet ($F(1,59)=41.64$; $p<0.001$) than those on the LF diet, without a line or interaction effect. The total energy content of feces assessed by bomb calorimetry was 35.3 % lower in HF-fed females than in LF-fed females ($F(1,59)=26.81$; $p<0.001$), but no line or interaction effect was observed. Furthermore, the relative energy content per feces mass was 10.5 % higher in HF-fed females than in LF-fed females ($F(1,59)=79.46$; $p<0.001$), again without a line or interaction effect. The absorbed energy was neither affected by diet nor line. However, the efficiency of absorption was significantly increased in mothers fed HF diet ($F(1,53)=44.38$; $p<0.001$), without any line differences.

Table 3. Energetics during peak lactation (day 13-16) of line 2 (control line), line 7 and line 8 (selected lines) mothers fed a low-fat (LF) or a high-fat (HF) diet.

	2 Control		7 Selected		8 Selected	
	LF	HF	LF	HF	LF	HF
Food intake (g)	60.1 ± 2.8	44.5 ± 3.6**	50.6 ± 3.2	40.6 ± 5.0	56.1 ± 3.5	51.2 ± 2.6
Food intake (kJ)	954.8 ± 45.3	875.1 ± 70.6	805.0 ± 50.5	797.6 ± 98.9	891.5 ± 55.7	1007.0 ± 51.0
RMR (kJ/h)	2.1 ± 0.1	1.6 ± 0.1**	1.9 ± 0.2	1.4 ± 0.1*	1.9 ± 0.1	1.7 ± 0.0
PIR (#/min)	1.6 ± 0.4	1 ± 0.2	3.7 ± 1.3	4 ± 0.8	2.9 ± 0.9	1.8 ± 0.9
Feces						
total dry weight (g)	7.9 ± 0.5	4.3 ± 0.5**	7.0 ± 0.6	3.6 ± 0.5**	7.5 ± 0.9	5.0 ± 0.6*
total energy content (kJ)	140.0 ± 7.3	83.4 ± 9.4**	124.0 ± 10.1	72.7 ± 10.2**	132.7 ± 15.1	101.6 ± 12.3
energy density (kJ/g)	17.7 ± 0.2	19.3 ± 0.3**	17.8 ± 0.2	19.6 ± 0.4**	17.9 ± 0.2	20.0 ± 0.2**
Absorbed energy (kJ)	814.8 ± 44.2	791.7 ± 62.4	681.0 ± 44.6	722.5 ± 89.2	758.8 ± 50.7	892.6 ± 46.4
Absorption efficiency (%)	85.0 ± 1.0	90.6 ± 0.5**	84.5 ± 1.0	90.7 ± 0.5**	85.1 ± 1.5	90.0 ± 1.1*

* denote significant effect of diet (*, $p<0.05$; **, $p<0.01$).

3.3.2. Growth efficiency of mothers and litters

Calculating weight change of the mothers combined with weight change of their respective litters over the course of peak lactation divided by the absorbed maternal energy over this period yields "growth efficiency" (GE) of mothers and pups. In Figure 3, GE is shown as averages in the different line and diet groups. GE was influenced by line ($F(2,49)=5.23$; $p<0.01$), diet ($F(1,49)=41.21$; $p<0.001$), and by an interaction between line and diet ($F(2,49)=4.53$; $p<0.05$). Thus, whereas effect of line on GE was variable when mothers were feeding the LF diet (i.e., with GE 24 % lower in line 7 and 21 % higher in line 8 relative to line 2), GE was significantly higher in line 7 and 8 animals feeding the HF diet than line 2 animals feeding the HF diet.

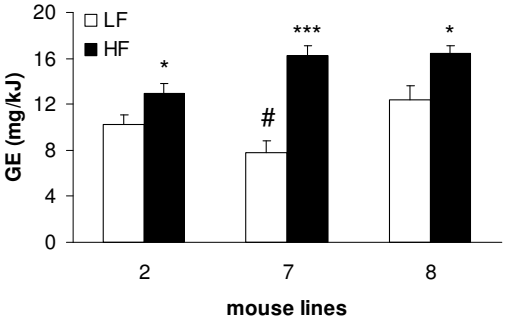

Figure 3. Growth efficiency (GE) of line 2 (control line), line 7, and line 8 (selected lines) mothers and their offspring fed a low-fat (LF) or a high-fat (HF) diet at peak lactation. # denotes significant line effect ($p<0.05$). *denotes significance difference with LF diet (diet effect) (*, $p<0.05$; ***, $p<0.001$).

3.4. Multiple regression analysis

It was reported previously that litter size is an important factor influencing (negatively) individual pup mass (e.g., Hayes et al., 1992), but it also might influence maternal investments in the offspring and therefore could affect ingestive behavior of the mother (Johnson, Thomson, and Speakman 2001a). Using multiple regression analysis, we investigated whether the relation between litter size and maternal and/or offspring traits were influenced by line and diet.

3.4.1. Litter size and mean pup mass

As expected and consistent with previous work of Johnson and colleagues (Johnson, Thomson, and Speakman 2001a), mean pup mass was correlated in a negative direction to litter size at birth. Within this correlation, pups of line 7 and line 8 weighed significantly less than those of line 2 at each corresponding litter size. HF diet did not cause any change in mean pup mass at birth at each corresponding litter sizes. From day 13 onwards, however, HF diet increased mean pup mass compared to those of LF-fed mothers at each corresponding litter size, and this effect was still observed at day 21.

Litter size remained negatively correlated to mean pup mass at day 13, 16, and 21. Furthermore, at these time points, line 7 and line 8 pup masses interacted positively with litter size, meaning that line 7 and line 8 pup masses were only smaller than those of line 2 pups in relatively small but not in large litters independent of diet. The interactions are shown for day 16 (peak lactation) in figure 4, which are exemplary for days 13 and 21. For intercepts, slopes, and p-values at the four time points of the lactation period, see Table 4.

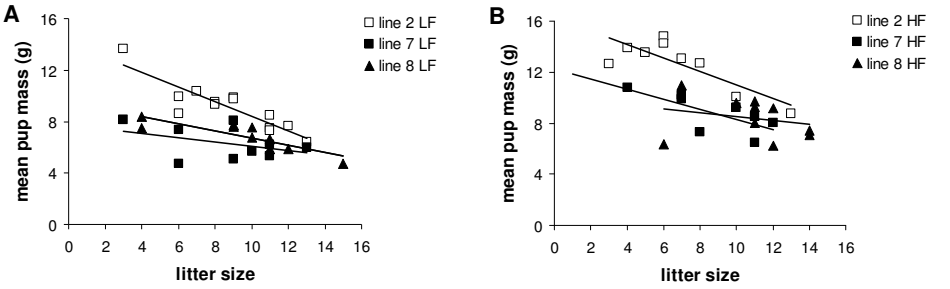

Figure 4. Relations between litter size and mean pup mass of line 2 (control line), line 7, and line 8 (selected lines) mothers fed a low-fat (LF, panel A) or a high-fat (HF, panel B) diet at peak lactation. Least-squares linear regressions are shown overall in LF diet condition: R^2=0.31, p=0.001; and in HF diet condition: R^2=0.49, p<0.001.

Table 4. Multiple regression analysis of litter size in relation to mean pup mass and litter weight at day 1 (birth), day 13, day 16, and day 21 (weaning) in interaction with line and diet.

	day 1		day 13		day 16		day 21	
	B	p	B	p	B	p	B	p
MEAN PUP MASS								
Intercept	2.21	<0.001	12.76	<0.001	13.94	<0.001	19.42	<0.001
littersize	-0.04	<0.001	-0.50	<0.001	-0.55	<0.001	-0.79	<0.001
Diet	-0.26	0.040	1.52	<0.001	2.50	<0.001		
Line 7	-0.17	<0.001	-6.22	<0.001	-5.37	<0.001	-8.71	<0.001
Line 8			-6.06	<0.001	-4.89	<0.001	-6.57	<0.001
Line 7 x Diet								
Line 8 x Diet								
Line 7 x Litter size			0.39	0.001	0.29	0.028	0.47	0.001
Line 8 x Litter size	-0.02	0.001	0.41	0.002	0.32	0.010	0.42	0.031
Diet x Litter size							0.27	<0.001
Line 7 x Diet x Litter size								
Line 8 x Diet x Litter size								
LITTER WEIGHT								
Intercept	1.55	0.038	29.90	<0.001	29.23	<0.001	41.62	<0.001
littersize	1.58	<0.001	4.44	<0.001	5.33	<0.001	7.00	<0.001
Diet								
Line 7			-21.70	<0.001	-22.52	<0.001	-35.07	<0.001
Line 8	3.24	0.023	-18.15	<0.001				
Line 7 x Diet								
Line 8 x Diet								
Line 7 x Litter size								
Line 8 x Litter size	-0.46	0.001			-1.85	<0.001		
Diet x Litter size			1.56	<0.001	2.19	<0.001	2.17	<0.001
Line 7 x Diet x Litter size								
Line 8 x Diet x Litter size								

Intercept (B) is shown by main effects and the interaction terms between line and diet; slope is shown by the two- and three-way interaction terms with litter size. P indicates the level of significance.

3.4.2. Litter size and litter weight

If mean pup mass contributes to the weight of a litter, then one would expect that litter size is positively correlated to litter weight, and that was indeed the case at birth as well as during other time points over the period of lactation. Line 7 had smaller litter weights than line 2 at each corresponding litter size from day 13 onwards. Line 8 also had smaller litter weights than line 2 in interaction with litter size at birth and at day 16, meaning that litter weights of line 8 were smaller only at large litter sizes. Furthermore, at day 13, litter weights of line 8 were smaller than those of line 2 at each corresponding litter size. At day 21, differences between lines were lost. Diet alone did not contribute to this model, but there was a significant interaction between diet and litter size from day 13 onwards (see Fig. 5 and Table 4).

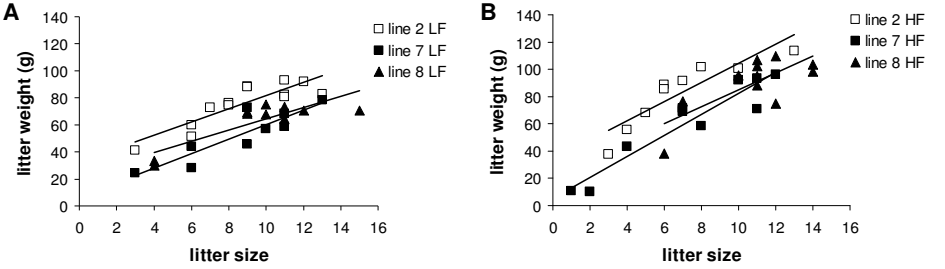

Figure 5. Relationship between litter size and litter weight of line 2 (control line), line 7 and line 8 (selected lines) mothers fed a low-fat (LF, panel A) or a high-fat (HF, panel B) diet at peak lactation. Least-squares linear regressions are shown overall in LF diet condition: $R^2=0.53$, $p<0.001$; and in HF diet condition: $R^2=0.69$, $p<0.001$.

3.4.3. Litter size and maternal energy absorption at peak lactation

As mentioned earlier, SusEI is maximized during the period of peak lactation, and the effect of energy procurement to each pup thus depends on litter size. The finding that mean pup mass was smaller only at small litter sizes but not at large ones in the selection lines makes it of interest to test relationships between litter size and absorbed energy. As expected, multiple regression analysis revealed a positive relation between absorbed energy and litter size (Table 5 and Fig. 6). Within this relation, feeding the HF diet caused the slope of the regression line to be more positive than for mice feeding the LF diet, which indicates that the absorbed energy was increased only at large litter sizes. Line 7 mice had significantly smaller absorption than line 2 at each corresponding litter size. Absorbed energy of line 8 was similar to that of line 2 in relation to litter size.

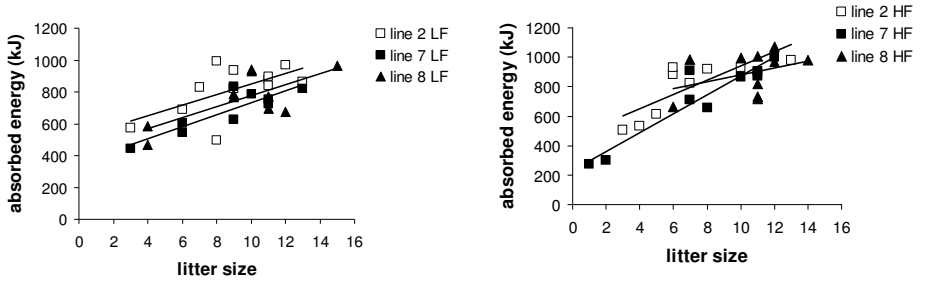

Figure 6. Relations between litter size and maternal absorbed energy of line 2 (control line), line 7 and line 8 (selected lines) mothers fed a low-fat (LF, left panel) or a high-fat (HF, right panel) diet at peak lactation. Least-squares linear regressions are shown overall in LF diet condition: R^2=0.48, p<0.001; and in HF diet condition: R^2=0.62, p<0.001.

Table 5. Multiple regression analysis between litter size and maternal/offspring traits (absorbed energy, combined weight gain of litter and mother, growth efficiency (GE) and Resting Metabolic Rate (RMR) during peak lactation in interaction with line and diet.

	Absorbed energy		Comb. weight gain		GE		RMR	
	B	p	B	p	B	p	B	p
Intercept	484.39	<0.001	5.76	<0.001	10.18	<0.001	1.54	<0.001
littersize	36.00	<0.001					0.07	<0.001
Diet							-0.38	<0.001
Line 7	-206.79	0.024			-2.43	0.029		
Line 8					10.42	<0.001	-0.24	0.025
Line 7 x Diet					4.93	0.002		
Line 8 x Diet								
Line 7 x Litter size							-0.03	0.025
Line 8 x Litter size					-0.85	<0.001		
Diet x Litter size	10.08	0.003	0.76	0.002	0.42	<0.001		
Line 7 x Diet x Litter size								
Line 8 x Diet x Litter size								

Intercept (B) is shown by main effects and the interactions term between line and diet; slope is shown by the two- and three-way interaction terms with litter size. P indicates the level of significance.

3.4.4. Interaction between litter size and combined weight gain of litter and mother

Litter size was not correlated with the combined weight gain of litter and mother over the course of peak lactation in the LF feeding condition (Table 5 and Fig. 7). However, the HF diet increased combined weight gain of litter and mother in interaction with litter size, meaning that combined weight gain was increased in the HF diet condition compared to the LF diet condition

only at large litter sizes. Combined weight gain of line 7 and line 8 was not different from that of line 2.

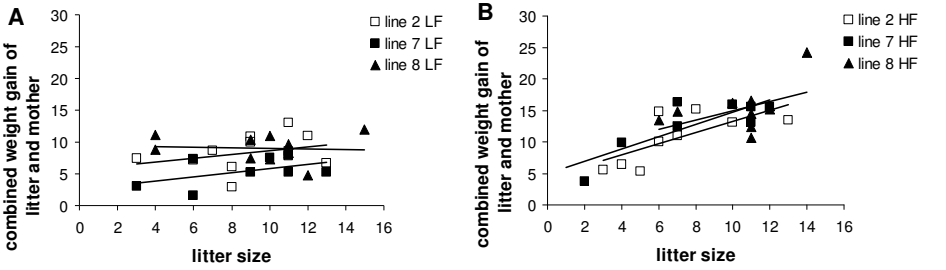

Figure 7. Relations between litter size and combined weight gain of line 2 (control line), line 7, and line 8 (selected lines) mothers and their litters fed a low-fat (LF, panel A) or a high-fat (HF, panel B) diet at peak lactation. Least-squares linear regressions are shown overall in LF diet condition: R^2=0.06, p=0.186; and in HF diet condition: R^2=0.57, p<0.001.

3.4.5. Interactions between litter size and GE at peak lactation

Litter size was not correlated with GE when animals fed the LF diet (Table 5 and Fig. 8). However, viewing lines separately, we observed that line 8 had a negative relationship with litter size, meaning that GE of line 8 was increased only at small litter sizes relative to that of line 2. Line 7 had smaller GE than line 2 at each corresponding litter size when fed a LF diet. Compared to the LF diet condition, HF diet caused an increase in GE only at large litter sizes, although the overall correlation was weak. Furthermore, HF diet caused line 7 to increase their GE to a larger extent than line 2 females and pups at each corresponding litter size.

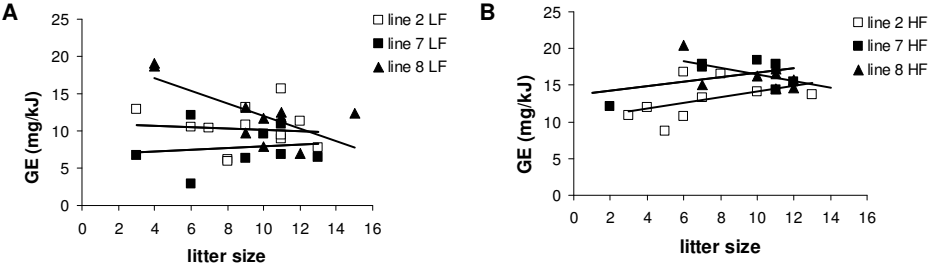

Figure 8. Relationship between litter size and GE of line 2 (control line), line 7 and line 8 (selected lines) mothers and litters fed a low-fat (LF, panel A) or a high-fat (HF, panel B) diet at peak lactation. Least-squares linear regressions are shown overall in LF diet condition: R^2=0.05, p=0.26; and in HF diet condition: R^2=0.17, p=0.039.

3.4.6. Interactions between litter size and RMR

Litter size was positively correlated with maternal RMR (Table 5 and Fig. 9). Line 7 showed an interaction with litter size, meaning that at large litter sizes RMR of line 7 mothers was lower than that of line 2 when fed the LF diet. Line 8 mothers fed the LF diet had lower RMR relative to line 2 at each corresponding litter size. HF diet decreased RMR at each corresponding litter size compared to the LF condition.

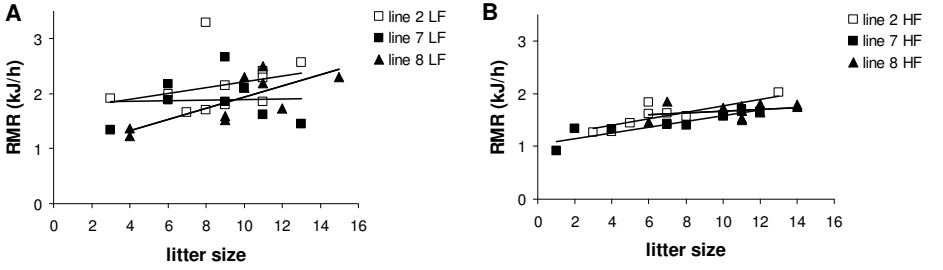

Figure 9. Relationship between litter size and resting metabolic rate (RMR) of line 2 (control line), line 7 and line 8 (selected lines) mothers fed a low-fat (LF, panel A) or a high-fat (HF, panel B) diet at peak lactation. Least-squares linear regressions are shown overall in LF diet condition: $R^2=0.14$, $p=0.049$; and in HF diet condition: $R^2=0.57$, $p<0.001$.

4. Discussion

The capacity to turn-over energy efficiently and at a high rate is an important asset to maintain a high level of physical activity over a long period. Finding nutrients under conditions of (anticipated) famine, or escaping increased levels of predation, for example, may rely on this capacity, and probably served as selective factors for many species. In many species, reproduction is a condition during which energy absorption and metabolic efficiency are increased, and often maximized. Rodents, for example, give birth to relatively large litters with a high growth rate, and subsequently face enormous energetic challenges during lactation (Johnson, Thomson, and Speakman 2001a; Johnson, Thomson, and Speakman 2001b). In the present study, we tested the hypothesis that a high capacity for physical activity and energy turn-over negatively affects a number of lactation characteristics, which we investigated in two mouse lines (designated line 7 and line 8) selectively bred for high voluntary wheel-running behavior and in a randomly bred control line (line 2) (see Swallow et al. 1998a) under conditions of feeding a low-fat (LF) or a high-fat/high-sucrose (HF) diet.

During pregnancy, line 7 and line 8 females were smaller than line 2 females - as under non-reproductive conditions – and also delivered pups with a lower birth weight. The number of newborn pups per litter, however, was increased in line 7 and 8 compared to line 2. While this

difference is largely consistent with a previous report on these activity-selected mice (Girard et al. 2002), a reduction in individual pup mass may be explained by energetic limitations intrinsically imposed by the size of the litter the pups were born in. As expected according to the work of Hayes et al. (1992), Johnson et al, and others, (Johnson, Thomson, and Speakman 2001a), mean pup mass was indeed correlated in a negative direction to litter size at birth in the present study. Within this correlation, however, pups of line 7 and line 8 weighed significantly less than those of line 2 at each corresponding litter size. Thus, selective breeding for increased physical activity has caused animals in these two lines to be smaller per sé - perhaps as an adaptation to limit incremental cost of locomotion (Rezende et al. 2006b) - rather than being the consequence of higher litter sizes in line 7 and line 8 mice relative to line 2. Diet effects on birth characteristics were less conspicuous, although a tendency was observed for lower litter sizes and weights in the HF diet condition in lines 2 and 7, but not in line 8, compared to the LF diet condition. Differences in vulnerability to HF-diet induced lipotoxicity may have caused line-specific effects (McCurdy et al. 2009).

Over the course of lactation, litter weight and individual pup mass became increased in the HF diet condition irrespective of line relative to the LF condition, despite the fact that absorbed energy was indistinguishable between diets and lines. As resting metabolic rate (RMR) of the HF diet feeding mothers was lower than of the LF feeding mothers at corresponding litter sizes, it is conceivable that more nutrients became available for lactation. This could have subsequently increased the flow of nutrients derived from dietary fat, presumably without first being processed by de novo lipogenesis in the condition of the LF diet (Rudolph, Neville, and Anderson 2007; Neville and Picciano 1997). Secondly, dietary fat causes lower thermic effects of ingestion than carbohydrate (Donato 1987), which allows more energy to be available for production of milk as well, and refined sugars are more easily absorbed. A consequence of this is that sustainable energy intake (SusEI) could have been elevated more easily without running the risk of lethal overheating, as suggested by the heat dissipation hypothesis of Krol and Speakman and colleagues (Krol and Speakman 2003). In line with this is the notion that above-mentioned effects of the HF diet to increase energy absorption and litter weight gain occurred specifically at large litter sizes, i.e., when SusEI would have been maximized sooner in the LF condition than in the HF condition.

When maternal energy absorption was taken into account during peak lactation (i.e., when energy intake by the mother reached a plateau between days 13 and 16), also growth efficiency (GE) of mother and pups was higher in the HF diet condition than in the LF condition. Interestingly, this effect appeared significantly stronger in both of the high-activity selected lines as compared with the control line. These data therefore imply that a trait for increased voluntary activity increases efficiency by which lactating females can procure energy for growth. At this point, we do not know whether the HF diet-induced exaggeration of GE in the high-activity lines was due to a more efficient mobilization of fuels for production of milk in the mammary tissue of the lactating mothers, to increased growth efficiency of line 7 and line 8 pups,

or both. RMR of the mother assessed by indirect calorimetry during peak lactation did not seem to play a role in the observed effects since the average values were affected by diet, but not by an interaction between line and diet. Furthermore, viewing RMR in relation to litter size only revealed differences between lines in the LF, but not in the HF diet condition, thus ruling out a potential role of this trait in the observed differences in GE. Under non-reproductive conditions, a physically active state is associated with increased energy requirements and fast nutrient mobilization and utilization (Hamilton and Booth 2000). This has also been supported by studies in selection lines having increased metabolic turn-over (Vaanholt et al. 2008) and increased aerobic capacity (Swallow et al. 1998). To facilitate these processes, there may be muscular enzymatic changes, which promote aerobic capacity (Rezende et al. 2006a; Vaanholt et al. 2008; Wong et al. 2009; Houle-Leroy et al. 2000). Provided that these differences exist in the reproductive state between the highly active animals and their controls, this might fuel mammary glands more optimally in the selected mothers, particularly in the HF condition. Studies in humans have shown that physical exercise can increase fertility and reproduction success by causing an increase in placental growth and vascularization only in the condition of obesity, i.e., a situation which might share some homology with the HF diet condition in the present study (Rich-Edwards et al. 2002; Weissgerber et al. 2006). Thus, animals with a trait for increased physical activity may redirect their high level of fat turn-over during the non-reproductive phase towards offspring development during lactation. This, however, does not rule out the possibility that line 7 and line 8 pups are more efficiently utilizing the nutrients obtained from the mother and add significantly to the increased growth efficiency of line 7 and line 8 animals relative to the line 2 animals.

Besides similarities in birth characteristics and GEs during peak lactation, a major difference between the two high-activity lines was that line 7 mothers, but not line 8 mothers, lost 37% of their offspring irrespective of diet. Perhaps the suppression of behavioral hyperactivity normally seen during lactation (Karasawa, Suwa, and Kimura 1981; Collier et al. 1984) was less effective in line 7 mothers than in line 8 and line 2 mothers, and this is indeed consistent with the higher PIR count during indirect calorimetry in the line 7 mothers. Physically active lactating mothers may be draining fuels away from milk production towards muscular activity (Speakman and Krol 2005). Such a mechanism is supported by our most recent observation that animals forced to work during lactation had increased pup loss and pups with stunted pup growth. One of the main differences among that studies and the current one is that selected animals in the present study were not forced to be active. We do not know at this point whether the increased voluntary and/or forced locomotor activity in these and other studies interfered directly with maternal care. At least, assessment of maternal care of these activity-selected mouse strains at generation 21 did not reveal differences in maternal care, but neither was there a difference in locomotor activity relative to the control lines when dams were housed without wheel access (Girard et al. 2002). Further studies must be performed to unravel the mechanisms of pup loss by line 7 mothers, as opposed to the other lines.

In summary, selection for high voluntary wheel-running activity generally favored increased growth efficiency (GE) when the mice were feeding a HF diet during pregnancy and lactation, but had variable effects when they were feeding a LF. The effects of the HF diet to promote GE occurred irrespective of reproductive success, as pup loss was variable among lines. It may be argued that the increased GE is adaptive for animals that need to cover a large habitat to fulfill nutritional requirements.

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CHAPTER 6

MICE SELECTIVELY BRED FOR HIGH VOLUNTARY RUNNING ARE RESISTANT TO PERINATAL PROGRAMMING OF WEIGHT GAIN BY A HIGH FAT DIET

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Summary

Feeding a high-fat/high sucrose (HF) diet during perinatal life programs offspring energy balance and may cause several health disturbing diseases associated with obesity at adulthood. However, a trait for high physical activity may balance and prevent these effects in the offspring. We hypothesized that selective breeding for high voluntary running in mice would protect offspring against these deleterious effects of perinatal HF feeding. Regression analysis, besides mean comparisons, was performed to investigate contribution of naturally occurring litter size in the effect of diet. At adulthood, HF diet caused proportional weight gain in male but not female offspring of the control line with elevated plasma insulin and low adiponectin levels. This was observed independent of the size of litter, but with a higher risk in small litter male offspring. Control female offspring from larger litter sizes developed elevated levels of glucose and cholesterol which might be the consequence of their increased food intake behavior. In contrast, male and female offspring of active lines became lean with elevated adiponectin levels and increased water intake, and particularly active female offspring developed lower levels of plasma leptin and insulin relative to controls. From these results, we conclude that male offspring are more susceptible to weight gain by the programming effects of HF diet. The trait for high voluntary running behavior in both genders reverses and/or defends against these effects and may provide a mechanism to prevent diet-induced weight gain.

1. Introduction

Locomotion enables animals to travel across the environment in the attempt to find partners, nutrients and fluids. In doing so, they need to combust extra fuels above the amount to maintain resting metabolic rate, in order to provide the energy for skeletal and cardiovascular muscular activity. The display of locomotor activity can vary tremendously between species, but also considerable individual differences can be observed within certain species (Scholz et al. 2008). In an extensive selective breeding program, Garland and colleagues assessed individual differences in running wheel activity of outbred mice, and created four replicate lines with high and four with normal locomotor activity (Swallow, Carter, and Garland, Jr. 1998). We and others have found that these high activity lines of mice have a number of metabolic and behavioral characteristics which may be viewed as adaptive to sustain increased physical activity levels in the natural environment (see Chapters 2, 3, and 4). From an energy balance point of view, these high activity mice are more resistant to develop diet-induced obesity (DIO) than control mice when subjected to feeding a high-fat (HF) diet at adulthood (Vaanholt et al. 2008). This is highly relevant in light of the view that dietary fat combined with a sedentary life-style is a frequent cause of human obesity in the modern industrialized societies (Dourmashkin et al. 2005; Lee and Korner 2008; Sanderson et al. 2008).

Besides studies dealing with regulation of energy balance at adulthood, also the perinatal origins of energy balance are becoming subject of intense investigation. Mechanism include maternal behavioral, hormonal and/or metabolic factors that can program fundamental regulatory systems in the fetal-newborn state, and this can subsequently influence the risk for attracting metabolic diseases in the offspring later in life (Levin 2006; Plagemann 2004; Plagemann 2005; Srinivasan and Patel 2008). One of the potential causal factors underlying the fetal origins of excess body weight at adulthood is early maternal overnutrition of dietary fat (Armitage, Taylor, and Poston 2005). Indeed, offspring from HF diet-induced obese female rats develop increased adiposity associated with cardiovascular and metabolic dysfunction indicative of the metabolic syndrome at adulthood (Samuelsson et al. 2008). Besides absorbed nutrients, also physical activity is an important determining factor in the energy balance equation (McMurray and Hackney 2005; Teske, Billington, and Kotz 2008). Physical activity is inversely related to the risk of attracting obesity (Patterson and Levin 2008; Tappy, Binnert, and Schneiter 2003), and it improves metabolic abnormalities even in the absence of weight loss in adults (Colberg 2007; Kruk 2007; Pearce 2008) as well in as children (Colberg 2007; Fogelholm 2008; Pearce 2008). In line with this view is the finding that already as little as 3 weeks of voluntary running wheel exercise at the juvenile stage is able to postpone obesity in diet-induced obese (DIO) male rats (Patterson, Dunn-Meynell, and Levin 2008). As mentioned earlier, mice selectively bred for voluntary high wheel running activity are shown to be resistant against HF DIO, even in the absence of running wheels (Vaanholt et al. 2008). Thus, while the trait for increased physical activity in mice is sufficient to maintain leanness by increasing metabolic rate and fat oxidation in the context of feeding a HF diet (see Chapter 3), it is unknown whether such

a trait also protects mice from developing disturbances in energy balance regulation when their mothers were fed a HF diet during pregnancy and lactation. Therefore, in the present study, selectively bred highly active mice and their randomly-bred controls were used to investigate the interactions between a physical activity trait and the effects of the peri-gestational HF diet feeding on several energy balance parameters in the offspring at adulthood, including body composition, food and water intake, and plasma levels of fuels and hormones. Since litter size is a major determinant of early growth as well (chapter 5 and (Johnson, Thomson, and Speakman 2001)), we investigated whether this factor also explained variations in the aforementioned energy balance parameters at adulthood of control and selected mice.

2. Materials and methods

2.1. Origin of animals and housing

Offspring of two selectively bred lines for high voluntary wheel running activity (line 7 and line 8) and of one randomly-bred control line (line 2) in generation 50 were used in these experiments. Parents of these mice were born to breeding couples within selected lines in their 48th generation, which were obtained from T. Garland Jr, Riverside, CA. For a detailed description of the selection procedure see (Swallow, Carter, and Garland, Jr. 1998). Selected lines were used for breeding without further selection at our facility in Haren. Mothers of offspring mice were fed either a standard low-fat (LF) lab chow diet (3.8 kcal/g; 58 % carbohydrate, 6 % fat, 22 % protein; Standard lab chow RMH-B 2181, HopeFarms BV, Woerden, NL) or a 40 % fat diet, additionally containing fast sugars (HF) (4.7 kcal/g; 30 % carbohydrate, 45 % fat, 18 % protein; AB Animal Diets, Woerden, NL) ad libitum 3 weeks before pregnancy until day 16 of lactation. After this period, the HF was replaced by the standard chow diet to avoid that the developing pups ingested the HF. This was necessary since mice start to ingest solid food after day 16. At birth, litter characteristics were assessed and litter sizes were kept non-manipulated (see Chapter 4).

2.2. Offspring and conditions

At weaning, offspring gender was determined and 3-4 mice of similar sex and perigestational diet were housed in standard cages (Macrolon Type II, UNO Roestvaststaal BV, Zevenaar, NL) with wood shavings and EnviroDry® bedding and they had ad libitum assess to standard lab chow diet and water. The room temperature was 22±1 °C with a 12:12 light-dark cycle (lights on at 8 am).

At 5-6 weeks of age, mice were characterized for running wheel activity over a 6 day period in similar type cages with bedding material and food and water available ad libitum, and with a running wheel (diameter: 14cm, code 0131 Savic®, Kortrijk, BE) attached to the side of the cage. The wheel revolutions were assessed by a computer, which collected data on a minute to minute basis. Data on running wheel activity over day 5 and day 6 were averaged, in the same

way as in the selection protocol. Right after the running wheel measurements, body mass was assessed. From each litter, two male and two female mice were blindly assigned to be included in these experiments, while the rest was used for other purposes.

Offspring included in this study were housed in couples of similar sex and from the same litter, with ad libitum access to food and water with the above-mentioned light, temperature and cage conditions. When they reached 4 months of age, food and water intake in each cage was assessed on two consecutive days, and was averaged per day per animal. Following food and water intake measurements, all offspring were anaesthetized by CO2, their nose-anal length was determined, and they were decapitated. Trunk blood was collected in tubes with Trasylol- EDTA, which then were centrifuged at 2600 g for 15 min at 4°C. Plasma was collected and stored at -80°C for later analysis of insulin, adiponectin, and leptin (analyzed by radioimmuno assays of Linco Research, Nucli lab, The Netherlands), and for cholesterol (enzymatic kit from Roche/Hitachi) and for glucose (by ferrycyanide method of Hoffman). Whole carcasses were kept without heads for total analysis. This included weighing of wet and dry masses (after drying to constant weight at 103°C for 24 plus 1 hour) and followed by fat extraction with petroleum ether in a soxhlet apparatus for 8 complete cycles. Thereafter, bodies were dried again to obtain dry-lean masses and calculate total fat- and fat-free mass (FFM).

2.3. Statistical analysis

Results were analyzed with GLM Univariate Analysis to test line and diet effects and their interaction in the measured parameters. Secondly, a Linear Regression Forward method was applied, which allowed us to investigate the contribution of litter size in the measured parameters. P value less than 0.05 was considered significant for all tests.

3. Results

3.1. Offspring characteristics at the adolescent stage

3.1.1. Running wheel activity

Between 6 and 7 weeks of age, all offspring animals were tested for running wheel activity. In figure 1, averages of running wheel activity on the 5th and 6th day of the 6-day subjection period are shown. In both genders, running wheel activity was significantly influenced by line (♂: $F(2,191)=141.3$; $p<0.001$, ♀: $F(2,188)=36.03$; $p<0.001$), but not by diet. Post-hoc analysis revealed that line 8 offspring ran significantly more than those of line 2 in both genders (♂: 2.9 times more, ♀: 1.7 times more). Males of line 8 also ran significantly more (2.3x) than those of line 7, but this effect was not observed in females. Females of line 7, on the other hand, ran 1.7x more than those of line 2, an effect not seen in males.

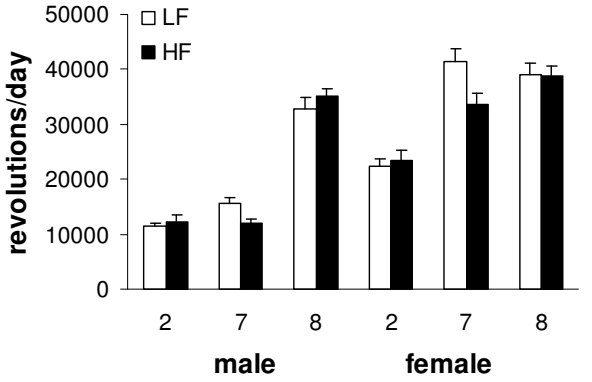

Figure 1. Average running wheel activity of male and female offspring of mothers perinatally fed LF and HF diet in line 2 (control line), line 7 and line 8 (selected lines) at 6-7 weeks of age.

3.1.2. Body weight

At the 7th week of age, body weight was significantly influenced by line in both genders (♂: $F(2,207)=93.5$; $p<0.001$; ♀: $F(2,204)=48.6$; $p<0.001$). Post-hoc analysis revealed increased body weight of line 2 males and females relative to those of line 7 and 8. Furthermore, body weight of line 7 female offspring was lower than that of line 8, an effect not observed in males. Body weight was also influenced by perigestational diet in males ($F(1,207)=51.7$; $p<0.001$), but not in females. Post-hoc analysis revealed that offspring of HF diet fed mothers had increased body weight relative to those of LF fed mothers. This effect appeared to interact with line ($F(2,207)=5.7$; $p<0.01$), meaning that line 2 and line 7, but not line 8 male offspring from HF fed mothers were found to be heavier than those of LF fed mothers (see table 1).

3.2. Offspring characteristics at the adult stage

3.2.1. Food and water consumption

At 4 months of age, food intake of offspring was significantly influenced by line ($F(2,80)=9.62$; $p<0.001$) in males, but not in females. Specifically, male offspring of line 8 consumed more food than those of line 2 and line 7. Perigestational HF diet had no effect on food intake (see Figure 2). In both genders, however, water intake was significantly influenced by line (♂: $F(2,79)=10.06$; $p<0.001$, ♀: $F(2,73)=19.85$; $p<0.001$) as well as diet (♂: $F(1,79)=7.57$; $p<0.01$, ♀: $F(1,73)=4.45$; $p<0.05$). Post-hoc analysis revealed increased water intake by line 7 and line 8 offspring relative to that of line 2 offspring, and offspring of HF-fed mothers consumed more water than that of LF-fed females irrespective of line (see Figure 3).

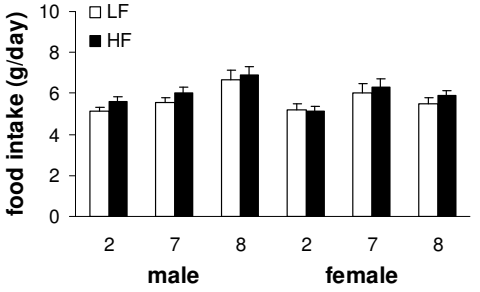

Figure 2. Food intake of male and female offspring of mothers perinatally fed LF and HF diet in line 2 (control line), line 7 and line 8 (selected lines) at 4 months of age.

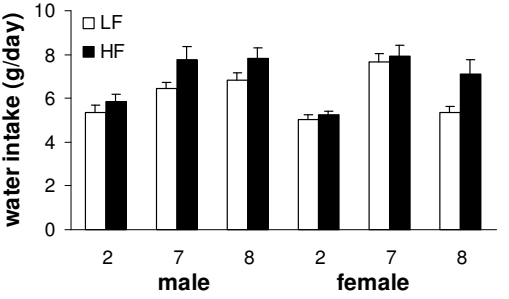

Figure 3. Water intake of male and female offspring of mothers perinatally fed LF and HF diet in line 2 (control line), line 7 and line 8 (selected lines) at 4 months of age.

3.2.2. Body weight and composition

At 4 months of age, offspring body weight was influenced by line in both genders (♂: $F(2,133)=23.28$; $p<0.001$, ♀: $F(2,130)=71.92$; $p<0.001$), and post-hoc analysis revealed that line 2 offspring was heavier than line 7 and line 8 offspring. Only in males, body weights were influenced by perigestational diet ($F(1,133)=13.41$; $p<0.001$), and increases were found in offspring of HF-fed mothers. Post-hoc analysis only revealed increased body weight in offspring from HF-fed mothers in line 2 (see Table 1).

Nose-anal length of offspring was influenced by line in both genders (♂: $F(2,130)=10.53$; $p<0.001$), ♀: $F(2,128)=37.81$; $p<0.001$). In line with the effects on body weight was the observation that line 2 offspring had higher nose-anal length than offspring of line 7 and line 8. Furthermore, nose-anal length was also influenced by perigestational diet ($F(1,130)=6.19$; $p<0.05$), but only in males; specifically male offspring of HF diet-fed mothers had higher nose-anal length than those of LF diet-fed mothers. Moreover, in males, an interaction between line

and diet ($F(2,130)=7.24$; $p<0.01$) was observed, and post-hoc analysis revealed that the nose-anal length of male offspring from HF diet fed mothers was increased only in line 2 (See table 1).

By absolute measures, drymass (♂: $F(2,132)=14.30$; $p<0.001$, ♀: $F(2,130)=65.31$; $p<0.001$), dry-lean mass (♂: $F(2,132)=22.11$; $p<0.001$, ♀: $F(2,130)=54.52$; $p<0.001$), body water content (♂: $F(2,132)=29.61$; $p<0.001$, ♀: $F(2,130)=47.47$; $p<0.001$) and body fat content (♂: $F(2,132)=12.70$; $p<0.001$, ♀: $F(2,130)=51.33$; $p<0.001$) were influenced by line in both genders. In general, these parameters were significantly increased in line 2 relative to lines 7 and/or 8 (see table 1). In addition, differences were found between lines 7 and 8 for dry mass (line 7 ♂ > line 8 ♂) and body water content (line 7 ♂ < line 8 ♂). Expressed as a proportion of body weight, %dry mass (♂: $F(2,128)=10.52$; $p<0.001$, ♀: $F(2,126)=46.51$; $p<0.001$), %dry-lean mass (♂: $F(2,128)=3.74$; $p<0.05$, ♀: $F(2,126)=20.37$; $p<0.001$), %body water (♂: $F(2,128)=10.13$; $p<0.001$, ♀: $F(2,126)=41.88$; $p<0.001$), and %body fat (♂: $F(2,128)=12.28$; $p<0.001$, ♀: $F(2,126)=45.15$; $p<0.001$) were all affected by line in both genders. Line 8 had the smallest %dry mass and %body fat and the highest %dry lean mass and %body water. In these respects, males of line 7 were similar to those of line 2, whereas females of line 7 were intermediate phenotype and significantly different from those of line 2 and line 8.

Perigestational diet influenced the absolute measures of dry mass ($F(1,132)=4.58$; $p<0.05$), dry-lean mass ($F(1,132)=9.68$; $p<0.01$), and body water content ($F(1,132)=17.16$; $p<0.001$) in males. In females, no effect of perigestational diet was observed. Post-hoc analysis revealed that above-mentioned parameters were increased in line 2 offspring of HF diet-fed mothers relative to those fed the LF diet. Body fat content was not influenced by diet in either gender when all lines were included in the analysis. However, when viewing line 2 males separately from lines 7 and 8, the HF diet condition significantly increased the level of body fat by 41%. When expressed proportionally to body weight, however, the perigestational diet effects were lost in male offspring, and was not observed in females either.

3.2.3. Metabolic fuel and hormone levels

Plasma insulin (♂: $F(2,131)=9.48$; $p<0.001$, ♀: $F(2,127)=11.64$; $p<0.001$), leptin (♂: $F(2,132)=8.74$; $p<0.001$, ♀: $F(2,130)=23.31$; $p<0.001$) and adiponectin levels (♂: $F(2,129)=6.44$; $p<0.01$, ♀: $F(2,129)=13.58$; $p<0.001$) were influenced by line in both genders. Post-hoc analysis revealed that offspring of line 2 had higher plasma levels of insulin and leptin, and lower plasma levels of adiponectin than those of line 7 and/or line 8 (see Table 2). Plasma glucose levels were also influenced by line ($F(2,127)=9.08$; $p<0.001$) but only in females; specifically, offspring of line 2 had higher levels of glucose than offspring of line 8. Furthermore, a line effect was revealed on plasma cholesterol levels ($F(1,129)=5.64$; $p<0.01$) in male, but not in female offspring; specifically, line 2 had lower cholesterol levels that those of line 7 and line 8. Perigestational diet had no effect on the above-mentioned fuel and hormone levels.

Table 1. Characteristics male and female offspring mice from line 2 (control line) and line 7 and line 8 (selected lines) mothers feeding either a LF diet or a HF diet during pregnancy and lactation.

	2		7		8	
	LF	**HF**	**LF**	**HF**	**LF**	**HF**
MALE						
Body mass						
7 weeks	30.5 ± 0.4	33.6 ± 0.4##	25.3 ± 0.5**	28.0 ± 0.4##	26.6 ± 0.4**	27.9 ± 0.3#
4 mnths	37.0 ± 0.6	40.7 ± 0.8##	34.0 ± 0.6**	35.9 ± 0.9	34.4 ± 0.7**	35.4 ± 0.7
Body composition						
Length	9.3 ± 0.1	9.9 ± 0.1##	9.1 ± 0.1	9.3 ± 0.1	9.2 ± 0.1	9.1 ± 0.1
Drymass	10.9 ± 0.3	12.8 ± 0.5##	10.3 ± 0.4	11.1 ± 0.9	9.6 ± 0.3**	9.4 ± 0.3
Drylean mass	8.0 ± 0.1	8.7 ± 0.2##	7.2 ± 0.1*	7.6 ± 0.2#	7.5 ± 0.1**	7.7 ± 0.2
Body water	21.2 ± 0.3	22.8 ± 0.4##	18.6 ± 0.3**	20.1 ± 0.4##	20.1 ± 0.4*	20.8 ± 0.4
Body fat	2.9 ± 0.2	4.1 ± 0.4	3.2 ± 0.3	3.4 ± 0.8	2.0 ± 0.2**	1.7 ± 0.2
% Dry mass	33.9 ± 0.5	35.8 ± 0.7	35.5 ± 0.8	34.9 ± 1.8	32.1 ± 0.5*	30.8 ± 0.6
% Dry-lean mass	25.0 ± 0.1	24.3 ± 0.3	24.8 ± 0.2	24.5 ± 0.3	25.5 ± 0.2	25.4 ± 0.3
% Body water	66.1 ± 0.5	64.2 ± 0.7	64.5 ± 0.8	65.1 ± 1.8	67.9 ± 0.5*	69.2 ± 0.6
% Body fat	8.9 ± 0.5	11.4 ± 0.8	10.7 ± 1.0	10.4 ± 2.0	6.7 ± 0.7*	5.4 ± 0.6
FEMALE						
Body mass						
7 weeks	25 ± 0.5	25.8 ± 0.6	19.6 ± 0.4**	21.6 ± 0.6	21.8 ± 0.4**	21.9 ± 0.3
4 mnths	34.2 ± 0.8	35 ± 1.5	26.7 ± 0.5**	27.6 ± 0.7	26.3 ± 0.5**	26.9 ± 0.6
Body composition						
Length	9.3 ± 0.1	9.4 ± 0.1	8.7 ± 0.1**	8.8 ± 0.1	8.6 ± 0.1**	8.5 ± 0.1
Drymass	11.1 ± 0.4	12.3 ± 1.0	7.8 ± 0.2**	7.6 ± 0.3	7.2 ± 0.3**	7.1 ± 0.2
Drylean mass	6.9 ± 0.1	6.8 ± 0.2	5.6 ± 0.1**	5.5 ± 0.1	5.6 ± 0.1**	5.7 ± 0.1
Body water	18.3 ± 0.4	18.0 ± 0.6	14.9 ± 0.3**	15.1 ± 0.3	15.2 ± 0.2**	15.5 ± 0.3
Body fat	4.2 ± 0.3	5.4 ± 0.9	2.2 ± 0.2**	2.1 ± 0.2	1.6 ± 0.2**	1.4± 0.1
% Dry mass	37.4 ± 0.6	39.8 ± 1.6	34.1 ± 0.5**	33.5 ± 0.6	31.9 ± 0.7**	31.1 ± 0.3
% Dry-lean mass	23.6 ± 0.3	22.8 ± 0.5	24.6 ± 0.1**	24.4 ± 0.3	25.1 ± 0.3**	25.2 ± 0.2
% Body water	62.6 ± 0.6	60.2 ± 1.6	65.9 ± 0.5**	66.5 ± 0.6	68.1 ± 0.7**	68.9 ± 0.3
% Body fat	13.8 ± 0.9	17.0 ± 2.0	9.5 ± 0.6**	9.0 ± 0.8	6.8 ± 0.9**	5.9 ± 0.4

Values given are means ± SEM. * denotes significant difference with line 2 (line effect) (*, $p<0.05$; **, $p<0.01$). # denotes significant difference with LF diet (diet effect) (#, $p<0.05$; ##, $p<0.01$).

4. Regression analysis

In chapter 4 it was observed that a number of growth and developmental pup characteristics were negatively correlated to litter size during lactation. An explanation for these correlations is that individual pups in relatively large litters received relatively less nutrients than those in

Table 2. Plasma hormone and fuel levels of 4 months old male and female offspring mice from line 2 (control line) and line 7 and line 8 (selected lines) mothers feeding either a LF diet or a HF diet during pregnancy and lactation.

	2		7		8	
	LF	**HF**	**LF**	**HF**	**LF**	**HF**
MALE						
Glucose (mM)	8.5 ± 0.3	8.1 ± 0.3	8.2 ± 0.4	7.5 ± 0.3	7.6 ± 0.3	7.8 ± 0.3
Insulin (ng/ml)	1.9 ± 0.2	2.7 ± 0.4	1.4 ± 0.2	1.6 ± 0.4	1.2 ± 0.2*	1.2 ± 0.2
Leptin (ng/ml)	1.8 ± 0.2	3.1 ± 0.5#	2.7 ± 0.4	3 ± 0.8	1.3 ± 0.3	1.1 ± 0.2
Adiponectin (µg/ml)	5 ± 0.4	4.8 ± 0.4	6.5 ± 0.5*	5.7 ± 0.6	6.9 ± 0.5**	5.9 ± 0.4
Cholesterol (mM)	2.6 ± 0.1	2.4 ± 0.1	3 ± 0.1*	3 ± 0.2	2.9 ± 0.2	3 ± 0.2
FEMALE						
Glucose (mM)	7.8 ± 0.3	8.2 ± 0.4	7.7± 0.2	7.0 ± 0.2#	6.6 ± 0.2**	7.1± 0.3
Insulin (ng/ml)	1.9 ± 0.3	2 ± 0.4	1 ± 0.1*	0.9 ± 0.2	0.9 ± 0.2*	0.8 ± 0.1
Leptin (ng/ml)	4 ± 0.5	4.5 ± 0.7	2.3 ± 0.3**	2.2 ± 0.4	1.3 ± 0.5**	1.1 ± 0.2
Adiponectin (µg/ml)	9.4 ± 0.8	9.5 ± 1.0	14.9 ± 1.4**	13.8 ± 1.2	16.8 ± 1.9**	15.7 ± 1.2
Cholesterol (mM)	1.9 ± 0.1	2 ± 0.2	2 ± 0.1	2.1 ± 0.1	1.8 ± 0.1	2 ± 0.1

Values given are means ± SEM. * denotes significant difference with line 2 (line effect) (*, $p<0.05$; **, $p<0.01$). # denotes significant difference with LF diet (diet effect) (#, $p<0.05$).

relatively small litters. Using multiple linear regression analysis, some of these "litter size-correlated pup characteristics" were line and/or diet specific. Here we investigated whether nutritional/energetic state parameters during adulthood were correlated to the size of the litter at peak lactation, and whether these correlations provided additional information above the statistical outcomes provided by mean comparisons.

4.1. Offspring characteristics at the adolescent stage

4.1.1. Running wheel activity

No overall correlation of running wheel activity with litter size was found, indicating that variation in litter size does not improve discrimination between groups.

4.1.2. Body weight

At 7 weeks of age, body weight of male and female offspring was negatively correlated to litter size, meaning that offspring mice reared in relatively large litters weighed less than those reared in relative small litters. Within this correlation, no overall effects of diet were observed, indicating that the effects of diet found by mean comparison are weakened when corrected for litter size. There was, however, an effect of diet in line 8 male offspring which appeared to interact with litter size (see Table 3), meaning that line 8 males born to HF diet fed mothers were smaller than line 2 males from HF diet fed mothers at large litter sizes, but not at small ones.

4.2. Offspring characteristics at the adult stage

4.2.1. Food and water consumption

Food intake was overall negatively correlated to litter size in female offspring, but no overall correlation was found in male offspring. Within this correlation, female offspring of HF diet fed mothers ate less than those of LF diet fed mothers. Since diet interacted positively with litter size in female offspring, this decreased food intake of female offspring of HF fed mothers occurred at small litter sizes, but not at large ones. Overall, water intake was not related to litter size.

4.2.2. Body weight and composition

Body weight at 4 months of age in males, but not in females, was negatively correlated with litter size. Within this correlation, line and diet effects were observed. First, male offspring from HF diet feeding mothers were heavier than those from LF feeding mothers at corresponding litter sizes. Secondly, line 7 and line 8 male offspring weighed less than those of line 2 mothers at corresponding litter sizes, irrespective of diet. Nose-anal length was negatively correlated with litter size at 4 months of age in both genders, with offspring from HF diet feeding mothers longer than those from LF feeding mothers at corresponding litter sizes, but this HF diet effect was only observed in males. Additional interactions showed that this correlation was significantly more negative in line 8 male and female offspring from HF diet feeding mothers relative to line 2 offspring, indicating that line 8 males and females were smaller than those of line 2, particularly when they were from large litters. In line 7 male and female offspring from LF feeding mothers, this correlation was less negative than found in line 2 offspring from LF feeding mothers irrespective of diet. None of the other body composition parameters were correlated with litter size.

4.2.3. Metabolic fuel and hormone levels

Plasma levels of adiponectin levels in males but not in females, were positively correlated with litter size. Within this correlation, plasma adiponectin levels were more negatively related to litter size in line 8 males than in line 2 males irrespective of diet. Furthermore, maternal HF diet feeding increased plasma adiponectin levels at each corresponding litter size in males irrespective of line. Plasma insulin levels in the male offspring were significantly correlated with litter size, but only in the HF diet condition, meaning that plasma insulin levels of male offspring from HF diet feeding mothers were only increased compared to those of LF feeding mothers at small litter sizes. In female offspring, on the other hand, plasma glucose and cholesterol levels were correlated with litter size only in the perigestational HF diet condition, meaning that plasma glucose and cholesterol levels were increased in female offspring from HF diet feeding mothers at large litter sizes, compared to female offspring from LF feeding mothers.

Table 3. Results of Regression Analysis, where B is the regression coefficient and p-level is the level of significance. Line and diet indicated as dummy variables, whereas litter size is the continuous independent variable. In the left column, Intercept represents the value of line 2 (reference group) crossing the y-axis and litter size represents the slope of the line representing the reference group (line 2). When line, diet and/or their interactions were found significant, the intercept of that particular group differed from line 2 (reference group). When an interaction with litter size was found significant, the slope of that particular group differed from line 2 (reference group).

	BW (7week)		BW (4 mon)		Body length		Drylean mass		% Drylean mass		Body water		Body fat		Insulin		Adiponectin		Cholesterol		Glucose		Food intake	
	B	p	B	p	B	p-level	B	p-level	B	p-level	B	p-level	B	p-level	B	p-level	B	p-level	B	p-level	B	p-level	B	p-level
MALES																								
Intercept	36.26	<0.001	40.99	<0.001	9.75	<0.001	8.68	<0.001			23.01	<0.001	3.06	<0.001	1.98	<0.001	2.76	0.009						
littersize	-0.59	<0.001	-0.39	0.002	-0.05	<0.001	-0.07	0.024			-0.19	0.045					0.23	0.030						
Diet			2.10	<0.001	0.50	<0.001	0.42	<0.001			1.17	<0.001	1.07	0.011	1.63	0.004	3.23	0.010						
Line 7	-5.5	<0.001	-5.90	0.003			-1.11	0.010			-3.25	0.006					1.70	0.001						
Line 8	-6.04	<0.001	-3.42	<0.001									-1.05	0.016	-0.81	0.004	5.07	<0.001						
Line 7 x Diet					-1.00	0.005																		
Line 8 x Diet	6.64	0.018																						
Line 7 x Litter size																								
Line 8 x Litter size							-0.06	<0.001									-0.31	0.033						
Diet x Litter size															-0.14	0.014	-0.36	<0.001						
Line 7 x Diet x Litter size																								
Line 8 x Diet x Litter size	-0.74	0.008			-0.05	<0.001							-0.12	0.028										
FEMALES																								
Intercept	30.17	<0.001			9.92	<0.001			20.12	<0.001									1.94	<0.001	7.74	<0.001	7.21	<0.001
littersize	-0.54	<0.001			-0.07	0.003																	-0.24	0.014
Diet									-0.35	0.049													-2.27	0.019
Line 7	-5.19	0.015			-1.18	<0.001			0.79	<0.001														
Line 8					-1.06	0.024			1.34	<0.001											-1.26	<0.001		
Line 7 x Diet																					-1.32	<0.001		
Line 8 x Diet																								
Line 7 x Litter size					0.07	0.024																		
Line 8 x Litter size																								
Diet x Litter size																			0.02	0.021	0.06	0.012	0.26	0.010
Line 7 x Diet x Litter size																								
Line 8 x Diet x Litter size					-0.10	0.032																		

5. Discussion

The aim of the present study was to investigate the effects of feeding a high-fat (HF) diet during pregnancy and lactation on offspring body weight gain and several associated parameters related to energy balance. This study was performed in a group of randomly bred control mice (line 2), and in two mouse lines that were from the same ancestral line as the controls, but were selectively bred for high voluntary wheel running behavior over 48 generations (Swallow, Carter, and Garland, Jr. 1998). We have previously observed that these highly active mice are hyperphagic but nonetheless resistant to diet-induced obesity (DIO), when fed a HF diet at adulthood. Control mice, on the other hand, were prone to develop DIO when subjected to a HF diet (Vaanholt et al. 2008). We hypothesized that the randomly bred female control mice, but not the highly active female mice, would program their offspring to exaggerated weight gain and related changes at adulthood when they would be fed a HF diet during pregnancy and lactation.

At 7 weeks of age, perigestational HF diet had an overall effect to increase body weight in male but not female offspring irrespective of line compared to the LF diet condition. This means that the trait for voluntary high-wheel running behavior did not protect the offspring against the stimulatory effect of perigestational HF feeding on body weight gain at the adolescent stage. Differences in running wheel activity between control and high activity lines were not influenced by the perinatal HF diet indicating that the HF diet did not program weight gain via behavioral inactivity. At the adult stage of 4 months of age, the stimulatory effect of perigestational HF feeding on body weight gain persisted in the highly active males irrespective of line. Viewing the data more closely, this effect of perigestational HF diet appeared to be skewed towards the control line, and was less pronounced in line 7 and even less so in line 8 males. Fluctuations in body weight can be the result of changes in fat-free mass, fat mass and/or body water. Analysis of these parameters revealed that particularly fat free mass and body water contributed to the increased body weights of the male offspring from HF diet fed line 2 mothers, and pointing out that these line 2 offspring from HF fed mothers were simply larger animals than the line 2 offspring from LF feeding mothers. This is reflected by the finding that also nose-anal lengths of line 2 offspring from HF feeding mothers were increased compared to the offspring from LF feeding mothers. Almost none of these parameters (except for the elevated water content and dry-lean mass content in the male line 7 offspring from HF fed mothers compared to the LF mothers) were significantly affected by the perigestational HF diet in the line 7 and 8 male offspring. Thus, it may be concluded that 1) male offspring is more susceptible to weight gain by perigestational HF diet than female offspring, and 2) programming effects of perigestational HF diet feeding are largely corrected in male offspring with a trait for increased voluntary wheel running behavior.

In previous studies, it was observed that feeding a HF diet during the perigestational period causes disproportional increases in adipose tissue (Lemonnier 1972; Samuelsson et al. 2008; Srinivasan et al. 2006). The finding in the present study that perigestational HF diet feeding did not cause an overall effect to increase fat mass is inconsistent with those previous

observations. This has two reasons. First, when only considering line 2 offspring, the perigestational diet caused a 41% increase in fat mass, which was not observed in line 7 and 8. Thus, the effect of perigestational HF feeding on adiposity was "missed" because of inclusion of all lines. Secondly, a strong disproportional increase in male line 2 offspring from perigestational HF fed mothers might have been ameliorated due the fact that the HF diet feeding mothers in the present study were switched back to the LF diet on day 16 of lactation, and the offspring were maintained on the LF diet as well. Most other studies investigating dietary programming effects on offspring energy balance left lactating mothers and progeny on the HF diet at least throughout weaning (see references above). The reason for the "early switch" was that we wanted to avoid direct effects of dietary fat on the offspring, and ended dietary treatment before pups have been observed to start ingesting solid foods (Kounig, Riester, and Markl 1988). Despite the absence of disproportional adiposity we found that plasma levels of insulin and adiponectin were increased in male offspring from HF feeding mothers relative to the LF feeding ones, irrespective of line. An elevation of plasma insulin has been shown previously to result from perigestational HF diet feeding (Parente, Aguila, and Mandarim-de-Lacerda 2008; Srinivasan et al. 2006), and together with the unchanged glucose levels this may be seen as an early sign of insulin resistance. An interesting phenomenon in light of the previous observations is that the perigestational HF diet feeding caused an increase in water intake, which was most pronounced in the high activity lines. In fact, the high activity lines had higher water intakes compared to the control line irrespective of diet. Since increased water intake stimulates cellular metabolism (Thornton, Even, and van Dijk 2009), it might be hypothesized that the high level of water intake is connected to the resistance of high activity lines to develop weight gain and potential metabolic disturbances induced by perigestational HF diet feeding.

Since litters of mice in the present study were not manipulated in size, it might be possible that naturally occurring differences in litter size among lines could have influenced body weights and associated parameters at adulthood. As mentioned in Chapter 5, sizes of litters the animals were born in were larger in lines 7 and 8 compared to line 2, and line 2 mothers feeding the HF diet tended to have even smaller litters than mothers feeding the LF diet. Although line 7 mothers had considerable pup loss during lactation, also litter size in this line tended to be lower in the HF condition than in the LF diet condition. In contrast, line 8 mothers feeding the HF diet tended to have even larger litters than in the condition of the LF diet. These differences between lines and diets might have contributed to differences in offspring energy balance parameters at adulthood. Indeed, it is known that extreme manipulation to very small (3-4 pups) or very large litters (18-20 pups) causes small litter offspring to become obese due to early overfeeding and large litter offspring to remain lean in the absence of overfeeding (Aubert, Suquet, and Lemonnier 1980; Plagemann et al. 1999). However, also relatively small differences of 2 pups less or more in the litter may contribute to differences in body weight parameters at adulthood (Epstein 1978). For example, observations in the study of Buckley et al. where litter size was reduced by perinatal HF diet from 15 to 12 probably contributed to the increased

adiposity of adult offspring from HF diet feeding mothers (Buckley et al. 2005). We therefore performed a Linear Regression Analysis including litter size, diet, line, as well as their interactions as independent factors and body weight and related parameters as dependent factor. We consistently observed in male, but also in female offspring that litter size contributed significantly in a negative direction to body weight at 7 weeks of age and only in male offspring at 4 months of age. Within this correlation in males, we observed that perigestational HF diet feeding contributed significantly to the increase in body weight at adulthood at each corresponding litter size, and line 7 and 8 males were smaller than line 2 offspring at each corresponding litter size. Furthermore, dry lean mass and body water content were also increased by perigestational HF feeding, and reduced in line 7 and 8 compared to line 2. These results are largely consistent with those obtained from the mean comparisons by GLM analysis.

The regression analysis between energy balance characteristics and litter size also revealed some novel effects that were not observed by mean comparisons in the GLM analysis. The level of body fat, for example, was not significantly increased by perigestational HF feeding in the overall mean comparison analysis, but was significantly elevated in the regression analysis by perigestational diet when ignoring line as a factor. Also line effects were found on the level of body fat, specifically line 8 male offspring had significantly less fat than line 2 males, irrespective of diet. Other effects found with regression analysis, which were not observed with mean comparisons related to differences in plasma hormone and fuel levels. Plasma levels of insulin in male offspring were negatively correlated to litter size, but only in the HF diet condition. The regression analysis revealed that differences between plasma insulin levels of HF and LF offspring was largest in small litters. Plasma adiponectin levels, on the other hand, were positively correlated with litter size in male offspring. Although plasma adiponectin levels within this correlation were increased somewhat at smaller litter sizes by perigestational HF diet feeding, these opposite correlations of insulin and adiponectin with litter size may have important consequences. Taking into account that the HF diet caused the largest weight gain in line 2 male offspring born in small litters, with the highest plasma insulin levels, but lowest plasma levels of adiponectin (despite some recovery as mentioned above), these animals were probably at highest risk to develop insulin resistance. The slight recovery of plasma adiponectin at small litter sizes by perigestational HF feeding might have protected them from developing type-II diabetes mellitus. Hence, adiponectin stimulates fat oxidation in skeletal muscle and contributes to glucose disposal and insulin action (Fruebis et al. 2001; Yamauchi et al. 2002), and reduces the risk of type-2 diabetes mellitus (Fruebis et al. 2001; Hara, Yamauchi, and Kadowaki 2005; Kadowaki et al. 2006). This is most relevant for offspring of line 2 since the high activity lines 7 and 8 have significantly elevated levels of plasma adiponectin. These latter effects are consistent with the findings in Chapter 4 and with previous work of Vaanholt et al. The final effects observed with regression analysis and not observed with mean comparisons related to plasma levels of glucose and cholesterol in female offspring, which were both positively correlated to litter size only in the HF diet condition. This means that the fuel levels were increased

independent of line in female offspring from HF feeding mothers at large litter sizes compared to offspring from LF feeding mothers. These effects may be related to food intake, since female offspring from HF feeding mothers ate more than those of LF feeding mothers at large litter sizes.

In summary, perigestation HF diet feeding caused weight gain in male offspring from randomly bred line 2 mothers, and this effect was largely absent in male offspring from the highly active line 7 and 8 mothers feeding the HF diet. Fat mass, fat-free mass as well as body water content appeared to contribute proportionally to the increased weight gain of the line 2 male offspring of HF fed mothers, and this effect was independent from litter size. Besides similarities between the high activity lines to resist effects of the perigestational diet, it was also clear that this resistance was strongest in line 8 offspring. It is likely that these differences results from the fact that the line 7 mothers lost an average 37% of pups in their litters during lactation, whereas line 8 mothers did not lose pups (see Chapter 5). The latter is remarkable since line 8 mothers had the largest litters among lines. It is therefore conceivable that individual pups in litters of line 8 mothers received less nutrition than individual pups in litters from line 7 mothers, and therefore remained the smallest and the lightest after lactation and at adulthood. Thus, even though line 7 offspring was relatively overnourished, the programming effects of perigestational dietary fat were probably offset by the genetic trait for increased wheel running behavior. Contributing mechanisms to this trait – besides increased voluntary activity and increased associated energy expenditure - could be related to the relatively high level of water intake, and a relatively high plasma level of adiponectin.

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CHAPTER 7

POSTNATAL VERSUS PRENATAL EFFECTS ON OFFSPRING ENERGY BALANCE REGULATION IN CONTROL MICE AND MICE SELECTIVELY BRED FOR HIGH VOLUNTARY WHEEL RUNNING

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Summary

Maternal behavior and nutritional factors have been shown to imprint neurobiological controls over energy balance in the offspring during postnatal life. This is particularly relevant in species that give birth to multiple pups at once, and are faced with nursing large litters where above-mentioned maternal factors can easily become limited for the individual kids. In these experiments, we investigated the effects of the postnatal influences in mice selectively bred for voluntary high running under low-fat (LF) and a high-fat (HF) feeding conditions. The activity selected lines generally give birth to relative large litters, but have mixed success in nursing their offspring, presumably due to the activity trait. Our aims were 1) to examine the effects of perigestational diet on maternal and offspring characteristics in equalized litter conditions, and 2) to investigate postnatal environment exchange between active and control lines by cross-fostering of pups. Firstly, we found that litter size at birth needs to be taken account in equalizing litter sizes. Manipulation with already two pups difference in the litter can result in contradictory effects, potentially due to a mismatch with expected number of pups the mothers prepared for. Secondly, control offspring fostered by active mothers resulted in smaller growth, lower glucose and insulin levels at adulthood of the fostered controls, meaning that the physical activity trait of mothers strongly influences the control offspring later in life. The opposite, in which selection line pups were fostered by control mothers did not have a major effect on adult energy balance regulation in these animals. Thus the prenatal, genetic make-up of activity selected offspring overpowered the relatively rich postnatal environment of control mothers, even when they were subjected to a HF diet. In conclusion, postnatal epigenetic effects seem to play only a limited role in the phenotype of mice selectively bred for high running wheel behavior.

1. Introduction

During the perinatal stage, metabolic, physiological and behavioral traits can be programmed which could affect maintenance of health and proneness for certain diseases later in life (Bouret 2009; Armitage et al. 2005; MohanKumar et al. 2007; McGowan, Meaney, and Szyf 2008; Barnes et al. 1966). It has been claimed for example that pregnant and lactating subjects eating high amounts of sugars and saturated fats adversely influence their offspring, with increased risk of developing obesity and related co-morbidities later in their lives. In our previous study in control mice, we consistently observed that feeding a 40% high-fat diet combined with refined sugars until day 16 of lactation, was able to increase weight gain and adiposity in the adult male offspring. These perinatal diet effects, however, were not observed in mice from the same ancestral line, but subsequently selectively bred for increased voluntary wheel running behavior over 50 generations. Programming effects of an unhealthy diet can apparently be offset by a trait for voluntary behavior. In that study, we also observed that the reproduction output of the selected and control animals differed, i.e., selected mice gave birth to on average larger litters than control mice. Large litters are generally comprised of relative small pups, while pups in relatively small litters tend to be larger. This could imply that individual pups from selected mothers are less well nourished than those of controls. It was also shown previously that energy intake of lactating mice becomes limited intrinsically, when they have to nurse too large litter, which additionally influences average pup growth and development (Johnson, Thomson, and Speakman 2001). Therefore, litter size could be a major postnatal contributing factor to programming of energy balance in the newborn and later life.

Perinatal under- and overnourishment has been studied by experimentally increasing and decreasing litter size (Schultze 1954). The quantity of nutrition in suckling animals indeed resulted in permanent effects on growth and metabolism as well as mental development. Overnutrition early in life as a result of relatively fewer pups in a litter is an important cause of obesity and related metabolic diseases in the adult mice (Aubert, Suquet, and Lemonnier 1980; Winick and Noble 1967; Plagemann 2006; Faust, Johnson, and Hirsch 1980), and may be a model for human disease as well. To study the contribution of litter size in the effects of perinatal HF diet feeding and the trait for increased physical activity in offspring body weight development and associated metabolic, hormonal, and behavioral parameters, we equalized litter sizes in control and high activity lines under the different diet conditions. We hypothesized that line differences would be maintained with respect to consequences of perinatal HF diet feeding on offspring.

Next we addressed whether perinatal diet/ line interactions on pup characteristics and subsequent body weight related factors at adulthood are due to genetic differences between lines, or caused by postnatal effects of mothers on pups. The technique of cross-fostering is generally used to distinguish between the influence of genes and the postnatal environment (Hager, Cheverud, and Wolf 2009). For this reason equalizing litter size was combined with cross-

fostering of pups a) between litters born to mothers of similar lines, or b) between litters of mothers born to different lines. These effects were investigated in the situation when mothers were either feeding a low fat (LF) carbohydrate rich diet, or when they were feeding a HF diet. When a parameter relevant to regulation of body weight is not altered by cross-fostering between lines, a genetic basis for this parameter inside the cross-fostered pup may be considered more persistent than the postnatal influence exerted via the mother.

2. Materials and methods

2.1. Animals and housing

Female mice 5-7 months of age were selectively bred for voluntary wheel-running behavior (the base population was the Hsd:ICR strain) in generation 49 and they were the siblings of the animals used in chapter 4 and 5. Their founders from generation 48 were obtained from T. Garland Jr, Riverside, CA, which were used for further breeding for 2 separate selection lines (line 7 and line 8) and one separate control line (line 2) at our facilities in Haren without further selection for wheel-running activity. For a detailed description of the selection procedure see (Swallow, Carter, and Garland, Jr. 1998). All female mice were individually housed in type C cages with wood shavings and EnviroDry® bedding with food and water *ad libitum*. The food was either a healthy fibered low-fat diet (LF) (3.8 kcal/g; 58 % carbohydrate, 6 % fat, 22 % protein; Standard lab chow RMH-B 2181, HopeFarms BV, Woerden, NL) for half of the animals or a 40 % fat diet, additionally containing fast sugars (HF) (4.7 kcal/g; 30 % carbohydrate, 45 % fat, 18 % protein; AB Animal Diets, Woerden, NL. Animals were housed in a room with a temperature of 22±1 °C on a 12:12 light-dark cycle with lights on at 8 am. Virgin female mice were paired with males from the same line, diet and generation. After 3 weeks of pairing, males were removed and totally 39 females gave birth and allowed to raise litters.

Handling of lactating mothers was exactly the same as it was described in Chapter 4 (see in details in Chapter 4). Briefly, maternal body mass and food intake were assessed daily throughout lactation. Feces produced over day 13-16 of lactation were collected to determined energy absorption. Growth Efficiency (GE) was calculated for body weight gain of mother and pups (i.e., expressed as mg body weight gain/ kJ food absorbed).

2.2. Equalizing of litters and cross-fostering

At the day of delivery, the size and the mass of the litter were assessed and the proportion of males and females in the litters were scored. Based on that outcome male as well as female pups were either A) not exchanged and left with their own mother, B) exchanged between mothers within the same line and diet, C) exchanged between the lines on the same diet, or D) exchanged within the same line on different diets. Exchanging of pups according to C) was never done between lines 7 and 8. Only when two or more litters were born within one day difference or

less, exchanging pups was allowed before the 3rd day of post-natal life, because after the 3rd day pup-mother bonding is does not occur anymore. In the process of exchange on the first post-natal day, the largest variations in litter size were first narrowed to 9-12, and manipulated to 10-11 on the second or third post-natal day. At day 8, all the litters were equalized to 10, if possible. Individual pups (recognized by sex and toe-clip) were weighed at day 8, 13, 16 and 21. Cages were cleaned at the time of weighing. During peak lactation, between day 13 and 16, feces were collected from bedding material for analysis of energy density. At day 16, HF diet was changed for chow (LF) diet, for the reason that offspring start to eat from food hoppers. At day 21, offspring were weaned and group-housed into 3-4 with their same sex littermates.

At 4 weeks of age, half of the own offspring that was raised by the mother that delivered them, were sacrificed for hormone and body composition analysis. At 6-8 weeks of age running wheel characterization was performed with body mass measurement. At 3 months of age, body mass of offspring was assessed again and they were individually housed for food and water intake measurements. At 4 months of age, they were sacrificed for body composition and hormone analysis (the procedures more in details in Chapter 5).

2.3. Statistical analysis

Data was analysed with General Linear Model Univariate Analysis with the exception of GLM Repeated Measures of maternal body weight and food intake over the course of lactation. Body masses of offspring during lactation were analyzed with ANOVA nested design where mothers were nested into line*diet interaction.

3. Results of mothers and litters at birth

3.1. Mother characteristics

3.1.1. Body mass during pregnancy and lactation

Before pregnancy the average body weights of line 2, 7, and 8 females on LF diet were respectively 30.9g; 24.0g; and 25.6g. On the HF diet these body weights were respectively 33.9g; 24.5g; and 27.8g. GLM Univariate Analysis revealed effect of line ($F(2,43)=14.11$; $p<0.001$), and post-hoc analysis revealed that line 7 and 8 females weighed less than line 2 females irrespective of diet. Over the course of pregnancy, body weight increases were found without effects of line (Figure 1).

During lactation (d1-d16), GLM Repeated measures revealed line effects ($F(2,34)=5.85$; $p<0.01$), where line 7 mothers had significantly lower body masses than line 2, and line 8 mothers appeared to have an intermediate body weight. Time interacted with diet ($F(15,525)=2.62$; $p<0.001$), meaning that HF fed mothers weighed significantly less over time (Figure 2).

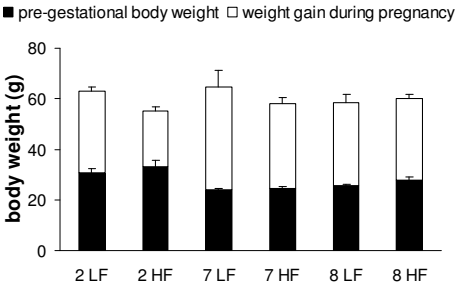

Figure 1. Pre-gestational body weight and weight gain during pregnancy in females fed LF diet (A) and HF diet (B) in line 2 (control line), line 7 and line 8 (selected lines).

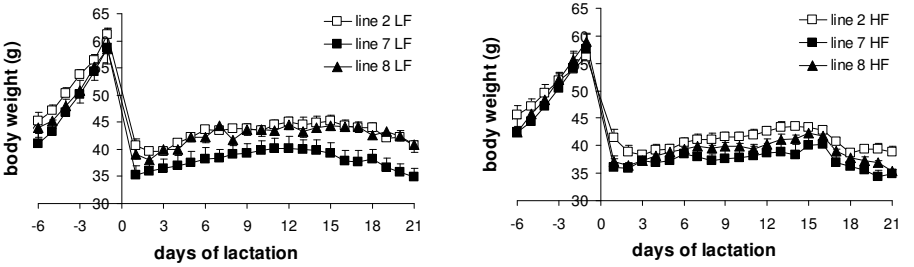

Figure 2. Body weight of females fed LF diet (A) and HF diet (B) in line 2 (control line), line 7 and line 8 (selected lines) during lactation.

3.1.2. Food intake during lactation

With repeated measures between day 1 and day 16 of lactation, a diet effect ($F(1,33)=5.67$; $p<0.05$), but not line effect was found on maternal energy intake (in kJ). Specifically, females feeding the HF diet increased their food intake compared to females eating the LF diet. There was an interaction between time and line ($F(28,476)=1.57$; $p<0.05$), time and diet ($F(14,476)=8.83$; $p<0.001$) and between time, line and diet $F(28,476)=1.79$; $p<0.01$), meaning that both line 7 and line 8 mothers increased their food intake when fed a HF diet, but not line 2. When the cumulative amount of food eaten during lactation was assessed from day 8 to day 16, an effect of line was revealed ($F(2,35)=3.70$; $p<0.05$), and post-hoc analysis showed that specifically line 8 females ate significantly more than line 7 females. Also an effect of diet was found ($F(1,35)=6.97$; $p<0.05$); i.e., mothers fed the HF diet ate more than those fed LF diet. (Figure 3).

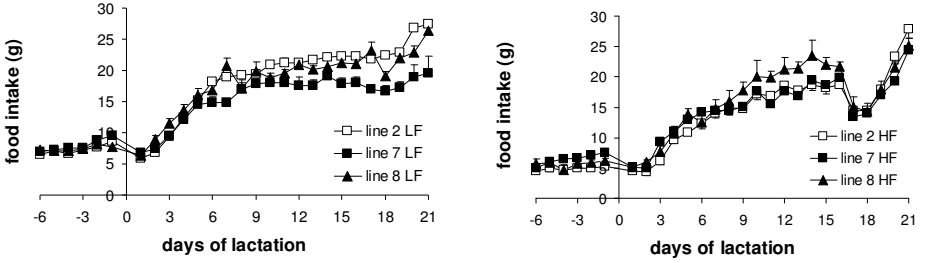

Figure 3. Food intake of females fed LF diet (A) and HF diet (B) in line 2 (control line), line 7 and line 8 (selected lines) during lactation.

3.2. Litter characteristics at birth

At birth, litter sizes and masses were assessed. We observed that line 8 had significantly larger litter sizes than line 2 ($F(2,38)=3.41$; $p<0.05$). Line 7 did not differ from either line (Table 1). Litter mass did not differ significantly between groups, however line 8 tended to have larger litter masses than line 2 ($F(2,38)=3.06$; $p=0.06$). Mean pup mass at birth did not differ between lines and diet conditions.

Table 1. Litter characteristics of mothers fed LF and HF diet during pregnancy and lactation in line 2 (control line), line 7 and line 8 (selected lines) at birth.

At birth	2		7		8	
	LF	HF	LF	HF	LF	HF
Litter size	11.8 ± 0.8	8.1 ± 1.0#	12.0 ± 1.3	11.5 ± 0.7	12.2 ± 1.2	13.3 ± 1.1
Litter mass	18.7 ± 0.8	13.6 ± 1.8#	19.6 ± 1.6	18.0 ± 1.7	19.3 ± 1.8	20.3 ± 1.0
Mean pup mass	1.6 ± 0.0	1.7 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	1.6 ± 0.0	1.5 ± 0.1

# denotes significance difference with LF diet ($p<0.05$).

Based on the outcome of the counted male and female pups, they were divided according to cross-fostering protocols A), B), C) mentioned earlier in the material and methods section. There were totally 3 litters in which adjusting the litter size to 10 was not possible, because of pup loss after day 3. These 3 litters were from one line 7 mother feeding HF diet (with 9 pups), from one line 8 mother feeding the LF diet (with 7 pups) and from one line 8 mother feeding HF diet (with 9 pups). Further details on the number of cross-fostered pups are provided in table 2 at pre-weaning. At post-weaning, the half of the own and all the cross-fostered offspring were used.

Table 2. Number of cross-fostered male and female pups raised by own, xf (condition B), line 2, line 7 and line 8 (condition C) mothers.

mothers	own	xf	7	8	own	xf	2	own	xf	2
	2LF offspring				7LF offspring			8LF offspring		
male	15	3	4	3	18	6	6	17	4	6
female	15	4	6	3	24	4	5	19	6	4
	2HF offspring				7HF offspring			8HF offspring		
male	8	4	3	3	13	3	3	25	4	7
female	9	4	4	5	12	4	5	15	4	3

4. Part A. Characteristics of own offspring

4.1. Body weight of own offspring

During the pre-weaning period, offspring characteristics were continuingly assessed at 8, 13, 16 and 21 days after birth. Using an ANOVA with a nested design, in which mother was a random factor and nested into line*diet interaction, males and females were analyzed separately. Mother attained a strong significant factor in each time point in the body mass differences of offspring during lactation in both genders. In general, maternal HF diet caused male and female offspring to be larger than those of LF diet fed mothers in each of the above-mentioned time points (d8: ♂: $F(1,92)=38.86$; $p<0.001$ and ♀: $F(1,89)=49.97$; $p<0.001$, d13: ♂: $F(1,92)=72.31$; $p<0.001$ and ♀: $F(1,89)=42.05$; $p<0.001$, d16: ♂: $F(1,92)=131.90$; $p<0.001$ and ♀: $F(1,89)=136.09$; $p<0.001$ and d21: ♂: $F(1,92)=57.03$; $p<0.001$ and ♀: $F(1,89)=61.47$; $p<0.001$) (see table 3). In males, line differences were revealed; i.e., at day 8 ($F(2,92)=8.27$; $p<0.001$) line 2 offspring were significantly heavier than that of line 7 and line 8. From day 13 onward (d13: $F(2,92)=13.27$; $p<0.001$, d16: $F(2,92)=17.07$; $p<0.001$, d21: $F(2,92)=30.27$; $p<0.001$), male offspring from different lines differed significantly from one another, with line 2 offspring being the heaviest and line 7 the lightest, and line 8 offspring of intermediate body weight. In the case of females, line effects were revealed only from day 16 onward (d16: $F(2,89)=17.46$; $p<0.001$, d21: $F(2,89)=22.92$; $p<0.001$), with line 2 offspring being the heaviest and line 7 the lightest with line 8 intermediate, similar to male offspring.

After weaning, body weights were assessed at 4 weeks, 6 weeks, at 3 months, and 4 months of age. At 4 weeks, maternal HF diet increased body mass in male ($F(1,39)=15.28$; $p<0.001$) offspring, but an effect in female offspring was lost. The HF diet effect was not attributed to the male offspring of line 2, but both line 7 and line 8 male offspring of HF feeding mothers were heavier than from LF feeding mothers (shown by interaction of line and diet). At 6 weeks of age, body mass was affected by line in both genders (♂: $F(2,52)=11.72$; $p<0.001$; ♀:

F(2,44)=12.06; p<0.001), but diet effects were lost. These effects were also observed at 3 months (line effects ♂: F(2,35)=4.70; p<0.05; ♀: F(2,41)=13.84; p<0.0001), and 4 months of age (line effects ♂: F(2,32)=6.71; p<0.01; ♀: F(2,36)=9.80; p<0.001). Specifically, line 2 offspring were heavier than those of line 7 and 8.

Table 3. Body weight of male and female offspring of mothers perinatally fed LF and HF diet during lactation in line 2 (control line), line 7 and line 8 (selected lines).

	2		7		8	
	LF	**HF**	**LF**	**HF**	**LF**	**HF**
MALE						
day 8	5.1 ± 0.1	5.8 ± 0.1	4.4 ± 0.1*	5.4 ± 0.2#	4.7 ± 0.2	5.2 ± 0.1
day 13	7.5 ± 0.2	9.2 ± 0.3#	6.3 ± 0.2*	8.2 ± 0.1##	7.0 ± 0.2	8.2 ± 0.2#
day 16	8.7 ± 0.2	10.9 ± 0.3##	7.1 ± 0.2**	9.7 ± 0.1##	8.0 ± 0.2	10.0 ± 0.2##
day 21	12.0 ± 0.3	14.3 ± 0.3#	9.1 ± 0.3*	11.6 ± 0.3##	11.2 ± 0.3	12.8 ± 0.4##
4 weeks	23.3 ± 0.5	23.3 ± 0.6	18.1 ± 1.0	22.1 ± 0.8#	17.0 ± 2.2	23.9 ± 0.6##
6 weeks	31.7 ± 1.0	32.3 ± 1.5	28.2 ± 0.6**	28.2 ± 0.6	30.4 ± 0.4	32.4 ± 0.8#
3 months	35.1 ± 0.7	35.9 ± 1.1	31.2 ± 0.8*	32.8 ± 1.5	34.1 ± 1.2	33.1 ± 0.9
4 months	40.6 ± 2.4	36.6 ± 0.9	33.9 ± 1.0*	33.7 ± 1.4	35.5 ± 0.9	34.9 ± 0.9
FEMALE						
day 8	4.8 ± 0.1	5.7 ± 0.2	4.2 ± 0.2*	5.4 ± 0.2	4.5 ± 0.1	5.0 ± 0.2
day 13	7.1 ± 0.1	9.5 ± 0.2##	6.2 ± 0.2	8.4 ± 0.1#	6.7 ± 0.1	8.1 ± 0.3#
day 16	8.3 ± 0.1	11.3 ± 0.2##	6.9 ± 0.2*	9.9 ± 0.2##	7.7 ± 0.2	10.2 ± 0.3##
day 21	11.3 ± 0.2	13.9 ± 0.4##	8.6 ± 0.4**	11.7 ± 0.2##	10.8 ± 0.3	12.8 ± 0.6
4 weeks	16.9 ± 1.5	15.4 ± 3.5	14.7 ± 1.1	17.3 ± 0.3	15.3 ± 0.7	18.4 ± 0.8#
6 weeks	25.3 ± 0.8	26.1 ± 1.5	20.5 ± 1.0**	22.1 ± 0.5	23.9 ± 0.8	26.3 ± 1.0
3 months	28.7 ± 1.0	28.5 ± 1.5	23.8 ± 0.5**	25.1 ± 0.4	26.2 ± 0.6*	28.2 ± 0.9
4 months	31.7 ± 1.2	31.6 ± 2.0	24.8 ± 1.3**	27.2 ± 0.4	27.5 ± 0.6**	29.6 ± 1.3

* denotes significant difference with line 2 (line effect) (*, p<0.05; **, p<0.01). # denotes significance difference with LF diet (diet effect) (#, p<0.05; ##, p<0.01).

4.2. Body composition of own offspring

Offspring cohorts of all groups were sacrificed and processed for body composition analysis at 4 weeks of age as well as at 4 months of age. At 4 weeks of age (see Table 4), body length was increased in male and female offspring by the maternal HF diet (♂: F(1,38)=6.56; p<0.05; ♀: F(1,34)=4.18; p=0.050), similar to the effects found on body weight, and this was attributed mostly to increases in line 7 and 8 offspring from HF diet fed mothers compared those from LF diet fed mothers. Furthermore, maternal HF diet increased dry mass (♂: F(1,38)=11.7; p<0.01; ♀: F(1,34)=5.10; p<0.05), dry-lean mass (♂: F(1,38)=9.3; p<0.01; ♀: F(1,34)=6,16; p<0.05) in both offspring genders, irrespective of line. Absolute levels of body fat (F(1,38)=11.1; p<0.01) as well as %body fat (F(1,38)=6.6; p<0.05) were increased only in male offspring from HF diet feeding mothers relative to offspring from LF diet feeding mothers, irrespective of line. Also

body water content ($F(1,38)=7.3$; $p<0.05$) was increased but %body water decreased in male offspring from HF diet feeding mothers relative to those from LF feeding mothers, and an interaction of line and diet revealed that effects of the peri-gestational diet on dry mass ($F(1,38)=3.2$; $p=0.052$) and body water content ($F(2,38)=3.5$; $p<0.05$) were mainly attributed to increased levels in lines 7 and 8, but not line 2 offspring. Female offspring of line 8 had increased dry mass ($F(2,34)=3.82$; $p<0.05$) and a tendency for increased dry-lean mass ($F(2,34)=3.28$; $p=0.052$) relative to line 2 female offspring.

With respect to the liver of 4 week old mice, male offspring of mothers on the HF diet had increased fresh liver weight ($F(1,38)=6.75$; $p<0.05$), liver dry mass ($F(1,38)=7.0$; $p<0.05$), liver dry-lean mass ($F(1,38)=7.6$; $p<0.01$) and liver water content ($F(1,38)=6.43$; $p<0.05$), effects not seen in female mice. Furthermore, compared to line 2 male offspring line 7 male offspring had livers with lower fresh weight ($F(2,38)=3.76$; $p<0.05$), dry mass ($F(2,38)=4.84$; $p<0.05$), dry-lean mass ($F(2,38)=4.06$; $p<0.05$) and water content ($F(2,38)=3.35$; $p<0.05$). The %liver dry lean mass ($F(2,34)=4.46$; $p<0.05$) was smaller in female offspring line 7 than that of line 2 offspring. On the other hand, liver fat content (♂: $F(2,38)=5.76$; $p<0.01$; ♀: $F(2,34)=4.97$; $p<0.05$) and %liver fat content (♂: $F(2,38)=3.55$; $p<0.05$, ♀: $F(2,34)=6.23$; $p<0.01$) was the higher in male and female offspring of line 8 relative to male offspring of line 7 and female offspring of line 2.

At 4 months of age (See Table 5), prior differences in body length caused by maternal HF diet were lost in both genders. The maternal HF diet increased %body water content in male offspring ($F(1,32)=6.30$; $p<0.05$), but none of the other body parameters in either gender was affected by maternal diet. In male offspring, maternal HF diet decreased dry-lean mass ($F(2,32)=3.73$; $p<0.05$) as well as total body water content ($F(2,32)=4.64$; $p<0.05$) in line 7 relative to line 2. An increased %body water content ($F(2,32)=4.16$; $p<0.05$) in line 8 male offspring was observed in male offspring from HF diet fed mothers relative to those of line 2 mothers. None of the other parameters reached significance. In female offspring, line effects revealed smaller dry mass ($F(2,36)=9.66$; $p<0.001$), fat mass ($F(2,36)=10.24$; $p<0.001$), %body water ($F(2,36)=6.04$; $p<0.01$), %dry-lean mass ($F(2,36)=3.70$; $p<0.05$) and %body fat ($F(2,36)=9.73$; $p<0.001$) in line 7 and line 8 relative to line 2 and in addition smaller dry-lean mass ($F(2,36)=8.22$; $p<0.01$) and body water ($F(2,36)=8.04$; $p<0.01$) in line 7 but not in line 8 relative to line 2.

Composition of the livers of 4 month old mice was not affected by diet. In general, effects of line revealed smaller liver fresh weight (♂: $F(2,37)=12.08$; $p<0.001$; ♀: $F(2,37)=6.04$; $p<0.01$), liver dry mass (♂: $F(2,37)=9.49$; $p<0.01$; ♀: $F(2,37)=6.96$; $p<0.01$), liver dry-lean mass (♂: $F(2,37)=7.25$; $p<0.01$; ♀: $F(2,37)=6.24$; $p<0.01$), liver water content (♂: $F(2,37)=12.60$; $p<0.001$; ♀: $F(2,37)=5.42$; $p<0.01$) and %liver water content (♂: $F(2,37)=3.4$; $p<0.05$; but not in ♀) mostly in line 7 male offspring relative to line 2 and line 8 male offspring. Liver fat content, %liver fat content and other % parameters were not affected by line or diet.

Table 4. Body composition of male and female offspring of mothers perinatally fed LF and HF diet in line 2 (control line), line 7 and line 8 (selected lines) at 4 weeks of age.

	2		7		8	
	LF	HF	LF	HF	LF	HF
MALE						
Body mass (g)	23.3 ± 0.5	23.3 ± 0.6	18.1 ± 1.0**	22.1 ± 0.8#	17.0 ± 2.2*	23.9 ± 0.6##
Length (cm)	7.9 ± 0.1	7.7 ± 0.1	7.3 ± 0.2	7.8 ± 0.2	7.1 ± 0.3	8.0 ± 0.1##
Drymass (g)	5.1 ± 0.1	5.1 ± 0.1	3.9 ± 0.3	4.9 ± 0.2#	3.6 ± 0.4	5.3 ± 0.2##
Dry-lean mass (g)	3.9 ± 0.1	3.8 ± 0.1	3.0 ± 0.2	3.7 ± 0.1#	2.8 ± 0.3	3.9 ± 0.2##
Water content (g)	12.4 ± 0.2	11.7 ± 0.4	9.4 ± 0.6	11.7 ± 0.4#	8.7 ± 1.0	12.0 ± 0.5##
Fat mass (g)	1.2 ± 0.1	1.3 ± 0.2	0.9 ± 0.1	1.2 ± 0.1	0.8 ± 0.1	1.4 ± 0.1##
%Dry-lean mass	22.2 ± 0.5	22.8 ± 0.4	22.4 ± 0.3	22.3 ± 0.2	22.8 ± 0.7	22.4 ± 0.2
%Water	71.1 ± 0.3	69.8 ± 0.9	70.8 ± 0.4	70.6 ± 0.5	71.1 ± 0.3	69.5 ± 0.5#
%Fat mass	6.7 ± 0.4	7.5 ± 1.2	6.7 ± 0.3	7.1 ± 0.6	6.1 ± 0.6	8.1 ± 0.5#
Liver fresh mass (g)	1.5 ± 0.0	1.5 ± 0.1	1.2 ± 0.1**	1.3 ± 0.1	1.1 ± 0.2*	1.5 ± 0.1#
Liver fat content (mg)	50.5 ± 3.4	47.4 ± 6.5	31.9 ± 2.4**	36.4 ± 4.8	37.7 ± 6.4	46.6 ± 2.2
%Liver fat content	3.4 ± 0.2	3.1 ± 0.3	2.8 ± 0.1*	2.7 ± 0.3	3.6 ± 0.5	3.2 ± 0.1
FEMALE						
Body mass (g)	16.9 ± 1.5	15.4 ± 3.5	14.7 ± 1.1	17.3 ± 0.3	15.3 ± 0.7	18.4 ± 0.8#
Length (cm)	7.4 ± 0.2	7.4 ± 0.3	6.9 ± 0.2	7.5 ± 0.1	7.1 ± 0.1	7.6 ± 0.1#
Drymass (g)	3.8 ± 0.3	3.4 ± 0.8	3.2 ± 0.3	3.8 ± 0.0	3.4 ± 0.1	4.9 ± 0.4##
Dry-lean mass (g)	2.9 ± 0.2	2.7 ± 0.4	2.4 ± 0.2	2.9 ± 0.0	2.5 ± 0.1	3.5 ± 0.3##
Water content (g)	8.6 ± 0.9	7.4 ± 1.7	7.4 ± 0.6	8.7 ± 0.2	7.4 ± 0.3	10.2 ± 1.0##
Fat mass (g)	0.9 ± 0.1	0.7 ± 0.3	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.4 ± 0.3#
%Dry-lean mass	24.1 ± 1.4	25.8 ± 1.6	22.6 ± 0.3	23.0 ± 0.3	23.4 ± 0.6	23.4 ± 0.8
%Water	69.3 ± 0.7	68.5 ± 0.4	69.9 ± 0.5	69.5 ± 0.5	68.8 ± 0.4	67.3 ± 1.5
%Fat mass	6.6 ± 1.0	5.7 ± 1.4	7.3 ± 0.5	7.5 ± 0.5	7.6 ± 0.8	9.2 ± 2.1
Liver fresh mass (g)	1.1 ± 0.1	0.9 ± 0.3	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.1	1.4 ± 0.1#
Liver fat content (mg)	27.3 ± 4.2	20.9 ± 10.9	27.4 ± 3.1	29.2 ± 2.2	35.1 ± 3.4	42.8 ± 7.3
%Liver fat content	2.4 ± 0.3	2.1 ± 0.6	2.8 ± 0.2	2.8 ± 0.2	3.4 ± 0.2	3.1 ± 0.2##

* denotes significant difference with line 2 (line effect) (*, $p<0.05$; **, $p<0.01$). # denotes significance difference with LF diet (diet effect) (#, $p<0.05$; ##, $p<0.01$).

4.3. Plasma hormone and glucose levels of own offspring

Analysis of blood samples taken from 4 month old mice revealed that maternal HF diet tended to decrease insulin levels in both genders (♂: $F(1,40)=4.06$; $p=0.052$; ♀: $F(1,37)=3.83$; $p=0.058$) and glucose levels in female offspring ($F(1,37)=3.82$; $p=0.059$), see table 6. In fact, these effects were attributed by line 2 offspring from mothers fed the HF diet. None of the other assessed circulating parameters were affected by diet. Line effects revealed low levels of insulin (♂: $F(2,40)=5.26$; $p<0.05$; ♀: $F(2,39)=4.87$; $p<0.05$) and leptin (♂: $F(2,40)=5.90$; $p<0.01$; ♀: $F(2,39)=11.74$; $p<0.001$) in male offspring of line 8 and female offspring of line 7 and line 8 relative to those of line 2. Glucose levels were also lower in line 7 and line 8 female ($F(2,37)=4.01$; $p<0.05$), but not in male offspring. Adiponectin levels were significantly different among all the three lines ($F(2,39)=15.03$; $p<0.001$) with line 8 having the highest and line 2 having the lowest levels of adiponectin and line 7 being intermediate.

Table 5. Body composition of male and female offspring of mothers perinatally fed LF and HF diet in line 2 (control line), line 7 and line 8 (selected lines) at 4 months of age.

	2		7		8	
	LF	**HF**	**LF**	**HF**	**LF**	**HF**
MALE						
Body mass (g)	40.6 ± 2.3	36.6 ± 0.9	33.9 ± 1.0*	33.7 ± 1.4	35.5 ± 0.9	34.9 ± 0.9
Length (cm)	9.7 ± 0.3	9.5 ± 0.1	9.4 ± 0.1	9.2 ± 0.2	9.2 ± 0.2	9.2 ± 0.2
Drymass (g)	9.6 ± 0.6	8.9 ± 0.4	8.6 ± 0.2	8.6 ± 0.5	8.6 ± 0.5	7.8 ± 0.3
Dry-lean mass (g)	7.6 ± 0.3	7.4 ± 0.2	6.9 ± 0.3	6.7 ± 0.2	7.1 ± 0.2	6.8 ± 0.1
Water content (g)	19.3 ± 0.3	20.0 ± 0.4	18.0 ± 0.6*	18.2 ± 0.8	19.4 ± 0.4	19.3 ± 0.4
Fat mass (g)	2.1 ± 0.5	1.5 ± 0.3	1.6 ± 0.3	1.9 ± 0.4	1.6 ± 0.4	1.0 ± 0.2
%Dry-lean mass	26.1 ± 0.9	25.7 ± 0.2	26.0 ± 0.7	25.2 ± 0.4	25.2 ± 0.3	25.1 ± 0.2
%Water	66.8 ± 1.4	69.2 ± 0.7	67.7 ± 0.6	68.0 ± 1.0	68.0 ± 6.8	71.3 ± 0.5
%Fat mass	7.1 ± 1.6	5.1 ± 0.8	6.3 ± 1.1	6.8 ± 1.2	6.8 ± 1.4	3.6 ± 0.5
Liver fresh mass (g)	2.2 ± 0.1	2.1 ± 0.1	1.7 ± 0.1**	1.8 ± 0.1	2.1 ± 0.0	2.1 ± 0.1
Liver fat content (mg)	80.0 ± 9.4	66.5 ± 6.5	53.3 ± 3.2*	64.8 ± 8.1	60.8 ± 6.3	72.4 ± 10.0
%Liver fat content	3.6 ± 0.4	3.1 ± 0.3	3.2 ± 0.2	3.6 ± 0.5	2.9 ± 0.3	3.5 ± 0.5
FEMALE						
Body mass (g)	31.7 ± 1.2	31.6 ± 2.0	24.8 ± 1.3**	27.2 ± 0.4	27.5 ± 0.6**	29.6 ± 1.3
Length (cm)	9.2 ± 0.1	9.1 ± 0.2	8.8 ± 0.2	8.8 ± 0.1	8.9 ± 0.1*	8.9 ± 0.2
Drymass (g)	9.4 ± 0.6	8.9 ± 0.9	6.3 ± 0.4**	6.9 ± 0.3	6.6 ± 0.2**	7.3 ± 0.8
Dry-lean mass (g)	6.0 ± 0.2	5.7 ± 0.4	5.0 ± 0.2**	5.2 ± 0.0	5.4 ± 0.1*	5.7 ± 0.2
Water content (g)	15.7 ± 0.5	15.5 ± 0.8	13.3 ± 0.7*	14.3 ± 0.2	14.5 ± 0.3*	15.7 ± 0.4#
Fat mass (g)	3.5 ± 0.5	3.2 ± 0.6	1.3 ± 0.3**	1.7 ± 0.3	1.2 ± 0.1**	1.6 ± 0.7
%Dry-lean mass	23.7 ± 0.4	23.6 ± 0.5	25.6 ± 0.7*	24.7 ± 0.3	25.8 ± 0.3**	24.7 ± 0.6
%Water	62.7 ± 0.8	63.8 ± 1.3	68.0 ± 0.9**	67.4 ± 1.2	68.7 ± 0.7**	68.6 ± 1.7
%Fat mass	13.6 ± 1.1	12.7 ± 1.7	6.3 ± 1.2**	7.9 ± 1.4	5.6 ± 0.6**	6.7 ± 2.2
Liver fresh mass (g)	1.9 ± 0.1	1.7 ± 0.2	1.4 ± 0.1**	1.6 ± 0.1	1.7 ± 0.1	1.8 ± 0.1
Liver fat content (mg)	107.6 ± 12.6	98.5 ± 5.6	71.0 ± 15.5	74.4 ± 5.6	76.7 ± 8.5	94.5 ± 15.7
%Liver fat content	5.7 ± 0.5	5.7 ± 0.5	4.8 ± 1.0	4.8 ± 0.3	4.6 ± 0.5	5.1 ± 0.6

* denotes significant difference with line 2 (line effect) (*, $p<0.05$; **, $p<0.01$). # denotes significance difference with LF diet (diet effect) (#, $p<0.05$).

4.4. Food and water intake of own offspring

Assessment and analysis of food intake by the offspring at 3 months of age revealed effects of diet in male offspring ($F(1,35)=6.12$; $p<0.05$), but no line effect was observed. Male offspring of HF diet feeding mothers ate significantly more than male offspring of LF diet feeding mothers, irrespective of line. In female offspring, a line effect ($F(2,41)=12.96$; $p<0.0001$), and a line*diet effect ($F(2,41)=5.35$; $p<0.01$) was observed on food intake. Specifically, female offspring of line 2 ate significantly less than line 7 and line 8 irrespective of diet, and female offspring of HF feeding mothers only had increased food intake in line 8. (see Figure 4).

In male offspring water intake revealed effects of diet ($F(1,35)=7.67$; $p<0.01$), but not of line. Specifically, male offspring of HF diet fed mothers drank more than offspring of LF diet fed mothers. In female offspring, however, water intake revealed a line effect ($F(2,41)=7.54$; $p<0.01$), but not a diet effect; with line 7 female offspring drinking more than 2 female offspring (see Figure 5).

Table 6. Hormonal and fuel levels in male and female offspring of mothers perinatally fed LF and HF diet in line 2 (control line), line 7 and line 8 (selected lines) at 4 months of age.

	2		7		8	
	LF	**HF**	**LF**	**HF**	**LF**	**HF**
MALE						
Glucose (mM)	9.4 ± 0.9	7.8 ± 0.5	8.5 ± 0.6	7.7 ± 1.0	6.9 ± 0.9	7.9 ± 0.6
Insulin (ng/ml)	1.7 ± 0.5	0.8 ± 0.3	1.2 ± 0.2	1.0 ± 0.2	0.6 ± 0.1	0.5 ± 0.1
Leptin (ng/ml)	2.5 ± 0.6	2.3 ± 0.5	3.0 ± 0.4	2.6 ± 0.6	1.8 ± 0.3	1.2 ± 0.2
Adiponectin µg/ml)	3.8 ± 0.6	4.5 ± 1.1	5.2 ± 0.4	4.7 ± 0.3	4.3 ± 0.6	4.3 ± 0.5
FEMALE						
Glucose (mM)	9.5 ± 0.8	7.5 ± 0.6	7.4 ± 0.4*	6.3 ± 0.5	7.1 ± 0.4**	7.4 ± 0.7
Insulin (ng/ml)	1.5 ± 0.4	0.7 ± 0.3	0.4 ± 0.2*	0.3 ± 0.1	0.6 ± 0.2*	0.3 ± 0.2
Leptin (ng/ml)	5.4 ± 1.0	4.3 ± 0.8	2.6 ± 0.6*	2.2 ± 0.3	1.6 ± 0.2**	1.9 ± 0.7
Adiponectin µg/ml)	8.3 ± 0.5	6.9 ± 1.0	9.6 ± 0.8	10.0 ± 0.8	11.2 ± 0.7**	12.7 ± 0.9

* denotes significant difference with line 2 (line effect) (*, $p<0.05$; **, $p<0.01$). No diet effect was observed.

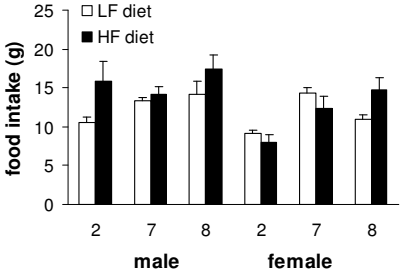

Figure 4. Food intake of male and female offspring of mothers perinatally fed LF and HF diet during lactation in line 2 (control line), line 7 and line 8 (selected lines) at 3 months of age.

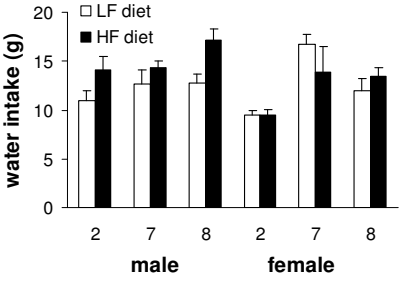

Figure 5. Water intake of male and female offspring of mothers perinatally fed LF and HF diet in line 2 (control line), line 7 and line 8 (selected lines) at 3 months of age.

4.5. Running wheel behavior of own offspring

Running wheel behavior was assessed according to the same methodology as used for selective breeding of lines. The average of the 5th and 6th day of total number of wheel revolutions was analyzed and since rodents are active in the dark phase, the dark phase of the 6th day was also examined specifically. In male offspring, running wheel behavior did not differ between lines and diet in either time period (see figure 6). In females, however, GLM revealed effect of line ($F(2,38)=4.20$; $p<0.05$), but not of diet. Specifically, line 8 offspring ran significantly more revolutions than line 2 offspring when the whole two days were examined. When only the dark phase was considered, both line 7 and 8 females ran significantly more than line 2 females ($F(2,38)=4.69$, $p<0.05$).

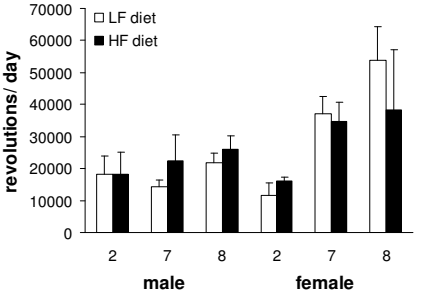

Figure 6. Average running wheel activity of male and female offspring of mothers perinatally fed LF and HF diet in line 2 (control line), line 7 and line 8 (selected lines) at 7 weeks of age.

4.6. Plus maze performance of own offspring

In male offspring, GLM Univariate Analysis revealed that maternal HF diet feeding increased the percentage of time spent in open arms ($F(1,48)=9.12$; $p<0.01$), the number of open arm entries ($F(1,48)=4.62$; $p<0.05$) and the percentage of open arm entries ($F(1,48)=10.42$; $p<0.01$). The percentage time spent in closed arms tended to be significant in the interaction of line and diet ($F(2,48)=3.14$; $p=0.053$). Closed arm entries were affected by line ($F(2,48)=3.28$; $p<0.05$), diet ($F(1,48)=7.09$; $p<0.05$), and by a line*diet interaction ($F(2,48)=4.25$; $p<0.05$). Specifically, offspring of HF diet fed mothers decreased their entries to closed arms, particularly in line 7, while offspring of line 8 was not affected. Percentage time spent in center area was affected by line ($F(2,48)=3.95$; $p<0.05$), diet ($F(1,48)=12.93$; $p<0.001$), and by a line*diet interaction ($F(2,48)=7.81$; $p<0.01$). Specifically, maternal HF diet feeding significantly decreased the time spent in the center, particularly strongly in line 7 offspring, while line 8 offspring were not affected. Total arm entries showed effect of line ($F(1,48)=3.64$; $p<0.05$); offspring of line 7 had increased total arm travels relative to offspring of line 2 (see Table 7).

In female offspring, GLM Univariate Analysis revealed that percentage of time spent in the center area was affected by line ($F(2,43)=7.10$; $p<0.01$), and by a line*diet interaction ($F(2,43)=7.01$; $p<0.01$). Specifically, offspring of line 2 spent significantly more time in the center relative to offspring of line 8, and this effect was amplified in line 2 offspring relative to lines 7 and 8 offspring from mother feeding the HF diet. While no effects were observed on time spent in closed or open arms, total arm entries in female offspring revealed effect of line ($F(2,43)=4.40$; $p<0.05$). Specifically, increased total arm entries of line 7 offspring relative to line 2 offspring were observed, with line 8 being intermediate. None of open arm or closed arm entries or percentage of open arm or closed arm showed difference among groups (see Table 7).

Table 7. Plus maze performance in male and female offspring of mothers perinatally fed LF and HF diet in line 2 (control line), line 7 and line 8 (selected lines) at adolescence age.

	2		7		8	
	LF	**HF**	**LF**	**HF**	**LF**	**HF**
MALE						
% Open time	13.6 ± 3.6	26.9 ± 12.9	4.2 ± 2.5*	16.7 ± 6.0#	8.6 ± 1.9	15.3 ± 1.8#
% Closed time	40.2 ± 3.6	49.4 ± 11.8	40.8 ± 4.6	51.3 ± 8.0	62.6 ± 3.9**	50.1 ± 4.0#
% Center time	46.2 ± 2.4	23.7 ± 3.0##	55 ± 3.9	32.1 ± 9.3#	28.9 ± 3.2**	34.7 ± 3.6
Open entries	4 ± 1.0	3.6 ± 1.3	2.3 ± 1.4	7.7 ± 2.1#	2.7 ± 0.6	4.3 ± 1.0
Closed entries	14.1 ± 1.5	9.2 ± 2.3	19.9 ± 1.9*	12.5 ± 1.7#	12.9 ± 1.1	14.3 ± 1.5
Total entries	18.1 ± 1.9	12.8 ± 1.5	22.2 ± 1.7	20.2 ± 3.7	15.6 ± 1.5	18.5 ± 1.8
% Open entries	21.4 ± 5.1	31.9 ± 12.4	9.9 ± 5.4	34.4 ± 4.1##	16 ± 2.6	22.6 ± 3.5
% Close entries	78.6 ± 5.1	68.1 ± 12.4	90.1 ± 5.4	65.6 ± 4.1##	84 ± 2.6	77.4 ± 3.5
FEMALE						
% Open time	8.5 ± 3.0	7.6 ± 4.4	10.2 ± 2.6	16.7 ± 6.9	13.6 ± 2.2	13.1 ± 2.8
% Closed time	51.4 ± 5.4	35 ± 5.2	45 ± 3.9	51.7 ± 5.8	53.8 ± 2.4	52.8 ± 3.4
% Center time	40.2 ± 3.8	57.4 ± 6.2#	44.8 ± 4.7	31.6 ± 1.5#	32.6 ± 2.4	34.2 ± 4.4
Open entries	3.4 ± 0.7	3.3 ± 1.6	5.2 ± 1.3	7.6 ± 2.2	4.7 ± 0.9	4.8 ± 0.7
Closed entries	15.3 ± 0.9	15.8 ± 2.8	18.2 ± 1.9	19.6 ± 2.1	15.8 ± 1.2	16.5 ± 1.3
Total entries	18.6 ± 1.3	19 ± 2.7	23.4 ± 2.9	27.1 ± 2.7	20.5 ± 1.2	21.3 ± 1.1
% Open entries	17.1 ± 3.1	16.7 ± 7.8	20.1 ± 3.9	26.3 ± 7.8	22.8 ± 3.7	23 ± 3.9
% Close entries	82.9 ± 3.1	83.3 ± 7.8	79.9 ± 3.9	73.7 ± 7.8	77.2 ± 3.7	77 ± 3.9

* denotes significant difference with line 2 (line effect) (*, $p<0.05$; **, $p<0.01$). # denotes significance difference with LF diet (diet effect) (#, $p<0.05$; ##, $p<0.01$).

5. Discussion on perinatal nutritional effects on own offspring

In a previous study, we observed that control mice subjected to a diet with an high fat (HF) and refined sugar content during pregnancy and lactation give birth to pups with increase growth rates and subsequently increased body weights at adulthood (see Chapter 4 and 5). This effect was found in males only. These data are seemingly consistent with many other studies

investigating the effects of western type cafeteria diets during the perinatal stage on programming of energy balance in the offspring (Lemonnier 1972; Parente, Aguila, and Mandarim-de-Lacerda 2008; Samuelsson et al. 2008; Srinivasan et al. 2006). We additionally found that the perinatal effects of feeding a HF diet did not result in increased growth rates in mice from the same ancestral line, but subsequently selectively bred for increased voluntary wheel running behavior over 50 generations. Programming effects of an unhealthy diet can apparently be offset by a trait for voluntary physical activity, even in the absence of running wheels. In that particular study, however, we observed that the number of pups per litter at birth of the selected mothers was significantly higher in the high activity selected mice than of the control line. Moreover, the HF feeding control mothers, but not the selected mothers, gave birth to fewer pups per litter than the low fat (LF) diet feeding controls. These effects were largely repeated in the present study. Since litter size is inversely related to individual pup weight (Chapter 4, (Johnson, Thomson, and Speakman 2001)) during lactation, and thus may be an important factor determining pup weight gain and development and subsequent body weight at adulthood (Chapter 5), a permissive role of reduced litter size in the perinatal HF diet effects on offspring body weight homeostasis can not be ruled out.

The mechanism underlying this effect is probably related to the fact that nutrient procurement of the mother to each extra pup is not entirely met by increased maternal nutrient intake (Schultze 1954; Johnson, Thomson, and Speakman 2001), which renders individual pups in large litters smaller than those in relatively small litters. In the present study, we aimed at investigation the consequences of equalizing litters of all mothers to 10 pups, and hypothesized that diet and line effects would be maintained despite equalizing litter sizes. Hence, adjusting litter size to 10 pups would cause line 2 HF feeding mothers to nurse two pups extra per litter, whereas it would cause line 7 mothers to nurse on average 1.5 and line 8 to nurse on average 3 pups less per litter. There is supporting data pointing out that even a difference of 2 pups additionally or less in the litter could cause a significant difference in body weight gain of offspring after weaning (Epstein 1978), which could indeed explain the observed effects in the previous chapters.

Consistent with our hypothesis was the observation that equalizing litter sizes to 10 caused individual pup weights of HF feeding mothers during lactation to be larger than those of LF feeding mothers, and secondly, differences between lines during lactation (i.e., with line 2 pups being heavier than line 7 and 8 pups) were maintained. While the HF feeding line 2 mothers were the only group nursing more pups than they were prepared for, their food intake was markedly reduced on the HF diet and remained behind that of line 7 and 8 females feeding the HF diet. It seems unlikely that the mothers of the control line feeding the HF diet were limited intrinsically by maximized heat dissipation, and unable to increase their food intake to nurse the additional two pups in the litter above the ones they received at birth. The argument for this idea is that dietary fat has a lower specific dynamic action than carbohydrates (Donato 1987), and this would allow higher energy intake before running the risk over over-heating (Krol

and Speakman 2003). At this moment, we have no explanation for this phenomenon. Line 7 and line 8 mothers, on the other hand increased their food intake, despite the fact that they nursed a lower number of pups relative to the number they were prepared for at birth. This provided extra procurement of nutrients from the HF diet feeding mothers to their pups, and probably amplified the HF diet effect leading to transiently increased body weights of line 7 and 8 offspring after weaning; i.e., an effect not seen in the unmanipulated litter condition of chapter 4 and 5. Because ingestive behavior of line 2 mothers feeding the HF diet did not compensate the two additional pups they were not prepared for, differences in offspring weight gain of HF- and LF feeding line 2 mothers were lost at weaning.

The lack of HF diet effects in line 2 controls, and the emergence of HF diet effects in line 7 and 8 at weaning - both phenomena contrasting with the findings in the unmanipulated litter condition in Chapters 4 and 5 and against our initial hypothesis- were largely reflected by body composition at 4 weeks of age. Thus, male offspring from HF diet fed line 7 and line 8 mothers had increased body fat content, lean mass, and body water compared to line 7 and 8 male offspring from LF feeding mothers. In line 2, male offspring from HF feeding and LF feeding mothers did not differ in body composition, and these effects differ from the unmanipulated litter condition in Chapters 4 and 5, where only line 2 HF feeding male offspring was heavier and had more body fat content that line 2 male offspring from LF feeding mothers. In female offspring in the present study, body fat content and body mass even increased in line 8 offspring from HF diet feeding mothers relative to the HF diet feeding line 2 mothers. Taken together, these data indicate that litter size appears to be an important factor in HF diet-induced alterations in body weight homeostasis at the pre-pubertal stage. Since line 8 females prepared for the largest litters, and thus nursed relatively fewest pups per litter, it is plausible to find the largest weight gain of individual pups at equalized litters in this particular line.

In the offspring at 4 months of age, diet effects on adiposity and lean mass were generally lost, while line effects became stronger, with line 7 and 8 male and particularly female offspring being smaller and leaner than line 2 male and female offspring. The hormonal profile of these animals very well fit these differences, with plasma leptin and insulin levels particularly lower in line 7 and 8 female offspring relative to those in line 2 offspring. In addition, increases were observed in plasma adiponectin levels in the high activity female offspring, reflecting the findings we had in previous studies (Vaanholt et al. 2007). These data demonstrate that perinatal effects of dietary fat content are limited on the long term in the offspring, even if initial body weight gain is in the opposite direction due to equalizing litter sizes. The trait for increased voluntary physical activity apparently overpowers pre-pubertal increases in body adiposity. Interestingly, food and water intake were increased in the male offspring from HF feeding mothers irrespective of line. This points out that perinatal HF diet feeding increased turn-over of nutrients specifically in male offspring, and may be related to early imprinting on neural circuits in the hypothalamus regulating energy fluxes. There are indeed supporting studies showing that early overfeeding or underfeeding either by diet or litter size manipulation caused permanent

changes in hypothalamic hormonal controls on food intake at adulthood (Davidowa, Li, and Plagemann 2003; Plagemann et al. 1999; Bouret 2009; Bouret and Simerly 2006). Other behavioral effects of perinatal HF feeding were decreased anxiety-like behaviors in male (i.e., increased time spent in the open arms in the plus maze test), but to a lesser extent in female (i.e., increased time in the center of the plus maze test) offspring during the plus maze performance test. Besides these effects, there were line effects to indicate that the offspring from the high activity lines showed more anxiety-like behavior and running wheel activity (in females only) than the control offspring, which is in agreement with the results of previous chapters.

In summary, energy supply, both qualitatively (maternal diet) and quantitatively (determined by litter size manipulation) during lactation have huge impacts on offspring development. Feeding a HF diet caused female mice in the control line to deliver smaller litters, but those in the selection lines to deliver the same or even larger litters than when they were feeding the fed LF diet. Equalizing litter sizes caused control females on a HF diet to nurse two pups more than they delivered, while selected lines nursed 1.5-3 pups less than they delivered irrespective of diet. Thus, control mothers faced increased requirements from their litters despite HF diet feeding, and this permanently diminished perigestational HF diet effects on body characteristics including weight, length, and adiposity. Selected mothers, on the other hand, were feeding less pups than they prepared for, which resulted in perinatal HF diet-induced obesogenic effects at the adolescent stage, particularly in line 8. These effects, however, were transient and overpowered by the physical activity trait at adulthood leading to smaller and leaner mice in lines 7 and 8 relative to lines 2.

6. Part B. Characteristics of cross-fostered offspring

Litter characteristics at birth were already described in paragraph 3.2 and shown in table 1. In this part, growth and development will be described of pups that were either cross-fostered by mothers within the same line and diet (condition B), or cross-fostered between lines 2 and 7, or between lines 2 and 8 within the same diet condition (condition C). Cross-fostering of pups between mothers on different diets within the same line was also performed (condition D), but yielded too few cases and is not described further. Table 2 shows numbers of cross-fostered and non cross-fostered pups in the different conditions. To assess whether body weight and related parameters are determined 1) by a genetic basis for these parameters inside the cross-fostered pup, or 2) by the postnatal influence exerted via the mother ("environment"), characteristics of cross-fostered offspring between lines (condition C) were compared to non cross-fostered "own" offspring (condition A; described in the previous part of this chapter). Effect of cross-fostering per sé was investigated by comparing non cross-fostered "own" offspring (condition A) with cross-fostered offspring between mothers within the same lines (condition B). Differences between offspring were analyzed with GLM Univariate Analysis according to comparisons mentioned above. Males and females were included in the analysis to investigate interactions between "environment" and "gender". Gender effects alone were not discussed.

6.1. Body weight of cross-fostered offspring before weaning

Before weaning, body masses of offspring were assessed at day 8, 13, 16 and 21. Before weaning, cross-fostering of pups between mothers within the same line on either diet never caused body weights of pups to differ from the non cross-fostered pups (comparison of condition A versus B). This means that the procedure of fostering was a negligible factor in adolescent growth.

Line 2 offspring from LF feeding mothers (further mentioned as 2LF, 7LF, etc) fostered by 7LF mothers yielded significant body weight effects from day 8 onwards (day 8 ($F(3,55)=6.94$; $p<0.001$), day 13 ($F(3,52)=23.20$; $p<0.001$), day 16 ($F(3,52)=26.48$; $p<0.001$) and day 21 ($F(3,52)=31.44$; $p<0.001$)). Specifically, 2LF offspring weighed significantly less when they were fostered by 7LF mothers than when they were raised by their own mothers, and in fact weighed less than 7LF own pups. Line 2LF offspring by fostered by 8LF mothers did not change body weight. Line 7LF offspring fostered by 2LF mothers significantly affected body weight from day 16 onward (d16: $F(2,63)=4.10$; $p<0.05$ and d21: $F(2,63)=3.39$; $p<0.05$). Specifically, 7LF offspring were heavier when they were fostered by 2LF mothers, but they weighed significantly less than 2LF own offspring (d16 and d21: $p<0.001$). Body weight of 8LF offspring was not altered due to fostering by line 2LF mothers or by the interaction with gender at any age. See Figure 7 left column.

In the HF diet condition, body weight of line 2HF offspring fostered by 7HF mothers caused significant effects on body weight only at day 21 ($F(3,44)=2.89$; $p<0.05$). Specifically, 2HF offspring weighed less when they were fostered by 7HF mothers, and in fact had similar weights as 7HF own offspring. No effect of fostering of 2HF offspring by 8HF mothers was found. From day 13 onward, body weight of 7HF (d13: $F(2,39)=6.03$; $p<0.01$, d16: $F(2,39)=8.61$; $p<0.001$ and d21: $F(2,39)=7.49$; $p<0.01$) as well as 8HF offspring (d13: $F(2,57)=4.70$; $p<0.05$, d16: $F(2,56)=4.60$; $p<0.05$ and d21: $F(2,47)=3.96$; $p<0.05$) fostered by 2HF mothers yielded significant effects. Both 7HF and 8HF offspring were heavier when they were raised by 2HF mothers compared to when they were raised by their own mothers, and in fact had similar weights as 2HF own biological offspring. See Figure 8 left column.

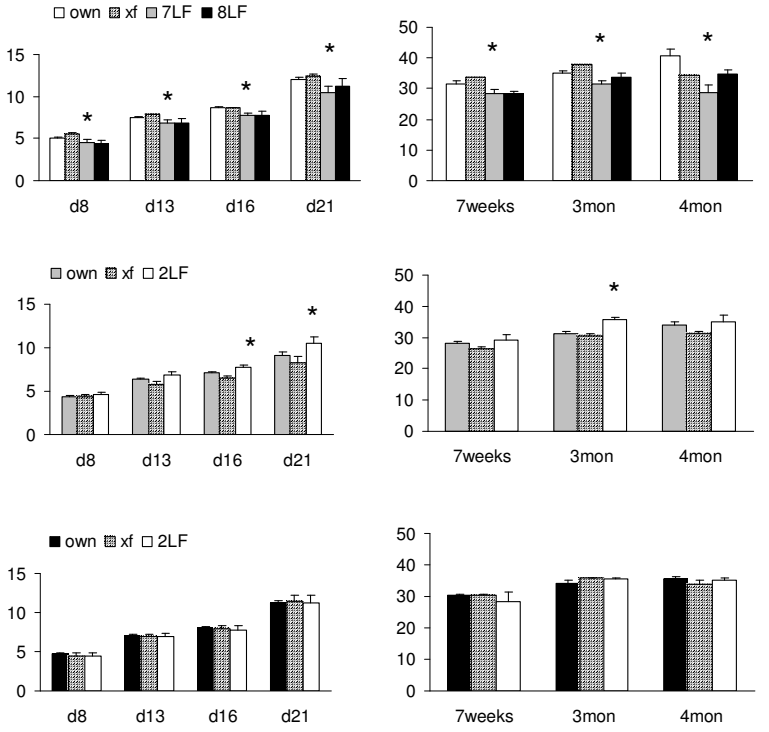

Figure 7. Offspring (males and females) body weight development described preweaning (left column) and postweaning (right column) when perinatally fed LF diet. Top raw shows 2LF offspring, middle raw shows 7LF offspring and bottom raw shows 8LF offspring raised by different mothers indicated in legend. * denotes significant difference between cross-fostered and own offspring ($p<0.05$).

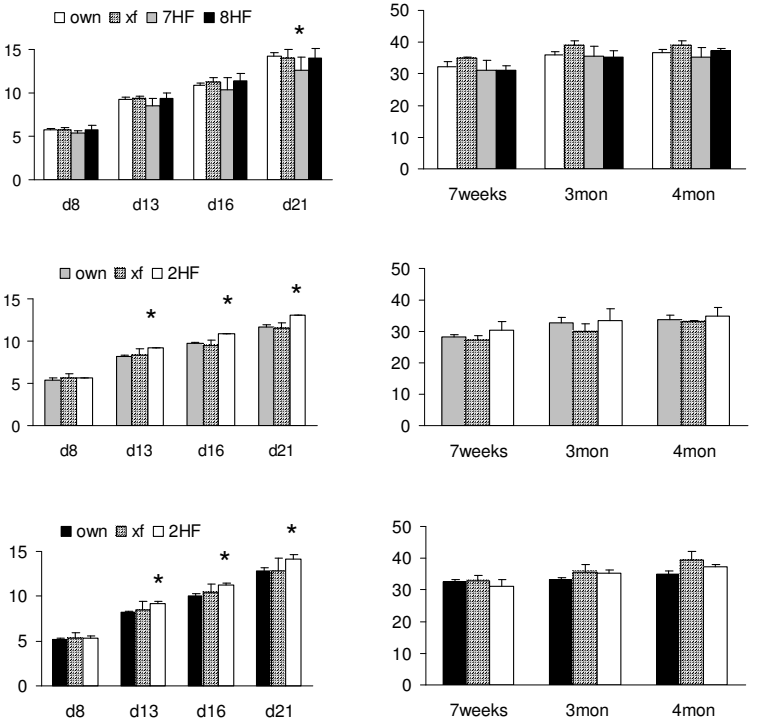

Figure 8. Offspring (males and females) body weight development described preweaning (left column) and postweaning (right column) when perinatally fed HF diet. Top raw shows 2HF offspring, middle raw shows 7HF offspring and bottom raw shows 8HF offspring raised by different mothers indicated in legend. * denotes significant difference between cross-fostered and own offspring ($p<0.05$).

6.2. Energy fluxes during peak lactation

During peak lactation (day 13-16), maternal food intake (g) was significantly influenced by line ($F(2,35)=3.38$; $p<0.05$) and by a line*diet interaction ($F(2,35)=3.49$; $p<0.05$); i.e., line 7 ate significantly less than line 8 mothers. When food intake was expressed as energy (kJ) effect of line ($F(2,35)=3.44$; $p<0.05$), diet ($F(1,35)=19.42$; $p<0.001$), and interactions of line and diet ($F(2,35)=3.41$; $p<0.05$) were revealed, and post-hoc analysis showed that mothers fed a HF diet ate more that those fed a LF diet, with the exception of line 2. Line 8 mothers ate more than line 7 mothers irrespective of diet (see Table 8).

The total amount of defecated waste during 3 days of peak lactation was significantly influenced by diet ($F(1,34)=12.33$; $p<0.01$), but not by line. Specifically, lactating females feeding HF diet defecated less than when they were feeding LF diet. The total energy content of the waste was also decreased by diet ($F(1,34)=5.99$; $p<0.05$). The waste energy content per unit

weight was significantly influenced by line ($F(2,34)=4.29$; $p<0.05$), diet ($F(1,34)=197.85$; $p<0.001$) and by a line*diet interaction ($F(2,34)=5.56$; $p<0.01$). Specifically, line 8 mothers had increased energy content in the feces per unit weight relative to line 2 mothers, and HF feeding also increased the energy content per unit weight.

Table 8. Energetic parameters during peak lactation (day 13-16) in females fed LF diet and HF diet in line 2 (control line), line 7 and line 8 (selected lines).

	2		7		8	
	LF	**HF**	**LF**	**HF**	**LF**	**HF**
Food intake (g)	66.5 ± 1.1	55.7 ± 2.3##	55.1 ± 3.1**	58.1 ± 5.4	62.8 ± 2.5	67.3 ± 4.1
Food intake (kJ)	1057.7 ± 18.0	1095.5 ± 45.1	875.6 ± 49.4**	1141.9 ± 106.4#	998.2 ± 39.7	1323.6 ± 80.6##
Feces						
total dry weight (g)	10.0 ± 1.8	6.0 ± 0.2#	6.3 ± 0.4	5.2 ± 0.3	8.4 ± 1.0	5.6 ± 0.3#
total energy content (kJ)	177.9 ± 33.1	115.9 ± 3.6	112.1 ± 7.5	106.1 ± 7.1	147.6 ± 17.6	116.5 ± 5.4
energy density (kJ/g)	17.7 ± 0.1	19.4 ± 0.3##	17.9 ± 0.2	20.2 ± 0.3##	17.6 ± 0.1	20.6 ± 0.2##
Absorbed energy (kJ)	879.8 ± 48.1	979.6 ± 43.1	763.5 ± 46.1	1035.9 ± 102.6#	850.7 ± 27.6	1207.2 ± 80.0##
Absorption efficiency (%)	82.9 ± 3.5	89.4 ± 0.4	87.1 ± 0.8	90.6 ± 0.6#	85.4 ± 1.4	91.1 ± 0.5##

* denotes significant difference with line 2 (line effect) (*, $p<0.05$; **, $p<0.01$). # denotes significance difference with LF diet (diet effect) (#, $p<0.05$; ##, $p<0.01$).

The energy absorption of the mothers was subtracted from the total ingested energy minus the total measured energy of the feces. This absorbed energy was higher in mothers fed a HF diet ($F(1,34)=24.3$; $p<0.001$). Absorption efficiency was increased by HF diet ($F(1,34)=14.69$; $p<0.001$). This energy was used by the mother itself and by the pups. Therefore to calculate the growth efficiency, the body weight gain of the mother and the pups was counted from day 13 to day 16 and both divided with the absorbed energy of the mother (mg/kJ). Growth efficiency was significantly influenced by line ($F(2,33)=4.16$; $p<0.05$), diet ($F(1,33)=258.10$; $p<0.001$) and line*diet interaction ($F(2,33)=6.11$; $p<0.01$). Specifically, line 7 had smaller GE when fed a LF diet. HF diet significantly increased GE with the exception of line 8. (Figure 9).

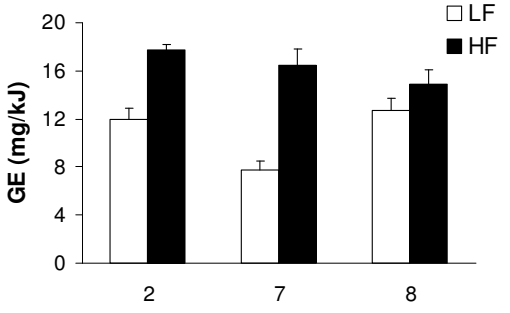

Figure 9. Growth efficiency of females fed LF and HF diet in line 2 (control line), line 7 and line 8 (selected lines) at peak lactation.

6.3. Body weight of cross-fostered offspring after weaning

Similar to the pre-weaning period, cross-fostering of pups between mothers in either line on either diet did not cause body weights of pups to differ from the non cross-fostered pups (comparison of condition A versus B).

Body weight of line 2LF offspring fostered by 7LF mothers yielded significant effects throughout the postweaning period to adulthood (at 6 weeks of age ($F(3,20)=7.30$; $p<0.005$), at 3 months of age ($F(3,20)=3.88$; $p<0.05$) and at 4 months of age ($F(3,23)=8.73$; $p<0.0005$)). See Figure 7 right column. Specifically, 2LF offspring weighed less when they were fostered by 7LF mothers compared to when they were raised by their own mothers, and in fact were indistinguishable from 7LF own offspring. Body weight of 7LF offspring fostered by 2LF mothers yielded interaction effect of environment and gender ($F(2,27)=4.49$; $p<0.05$) at 3 months of age but not at other time points. Thus at 3 months of age, 7LF male, but not 7LF female offspring were heavier when they were fostered by 2LF mothers and were indistinguishable from 2LF offspring. Body weight of 8LF offspring fostered by 2LF mothers was neither significantly by environment nor by the interaction with gender at any age. In the HF diet condition, neither body weights of 2HF, 7HF, nor of 8HF offspring were affected by cross-fostering. See Table 8 right column.

6.4. Body composition of cross-fostered offspring

Offspring body composition at 4 months of age is shown in Table 9. In the LF diet condition (Table 9A), 2LF offspring fostered by 7LF mothers yielded significant effects on body length ($F(3,23)=3.67$; $p<0.05$), dry-lean mass ($F(3,23)=4.28$; $p<0.05$), analogous to the effects on body weight. In none of the groups was body fat mass or %body fat significantly affected by cross-fostering. In stead, body length and dry-lean mass were smaller in 2LF offspring when they were fostered by 7LF, but not by 8LF mothers, and in fact indistinguishable from 7LF own offspring. When 7LF offspring were fostered by 2LF mothers, total body water content yielded an interaction effect of environment and gender ($F(2,28)=3.4$; $p<0.05$), but no other parameters were affected. Specifically, male offspring, but not female offspring, of 7LF mothers had increased body water content when they were fostered by 2LF mothers, and were indistinguishable from 2LF own biological offspring. Line 8LF offspring were not altered due to fostering by 2LF mothers. An effect of cross-fostering per sé was observed in 8LF offspring. Specifically, body water content ($p<0.001$) was increased in 8LF offspring by cross-fostering within lines.

In the HF diet condition (Table 9B), fostering of 7HF offspring by 2HF mothers caused significant effects on dry-lean mass ($F(2,17)=6.88$; $p<0.01$) and body water content ($F(2,17)=6.52$; $p<0.01$). Specifically, dry-lean mass and body water content were larger in 7HF offspring when they were fostered by 2HF mothers than when they were raised by their own mothers.

Table 9A. Body composition of mice selectively bred for high voluntary wheel running behavior (lines 7 and 8) and the control line raised by mothers they were born to ("own"), or when they were fostered by other mothers of the same line (xf), or by other mothers of other lines (xf2, xf7, or xf8). Males, females and LF- (A), HF diet (B) are shown in separate panels.

A	own	xf	7	8	own	xf	2	own	xf	2
	2LF offspring				7LF offspring			8LF offspring		
MALE										
BW	40.6 ± 2.3	34.4 ±	28.7 ± 2.6*	34.8 ± 1.2	33.9 ± 1.0	31.3 ± 0.5	35.2 ± 1.9	35.5 ± 0.9	33.9 ± 1.3	35.2 ± 0.6
Length	9.7 ± 0.3	9.3 ±	9.1 ± 0.2	9.1 ± 0.1	9.4 ± 0.1	9.5 ± 0.0	9.4 ± 0.3	9.2 ± 0.2	8.8 ± 0.3	9.4 ± 0.1
Drymass	9.6 ± 0.6	9 ±	7.7 ± 0.8	9.2 ± 1.0	8.6 ± 0.2	8.7 ± 0.4	9.5 ± 1.0	8.6 ± 0.5	8.9 ± 0.7	9.6 ± 0.3
Drylean	7.6 ± 0.3	8.3 ±	6.6 ± 0.4	7.3 ± 0.4	6.9 ± 0.3	6.7 ± 0.1	7.5 ± 0.4	7.1 ± 0.2	7.2 ± 0.4	7.5 ± 0.1
Water	19.3 ± 0.3	20.7 ±	16.3 ± 2.1	20.7 ± 0.6	18 ± 0.6	17.9 ± 0.4	20.7 ± 0.9*	19.4 ± 0.4	20 ± 0.7	20.8 ± 0.3*
Fat	2.1 ± 0.5	0.7 ±	1.2 ± 0.4	1.9 ± 0.6	1.6 ± 0.3	2 ± 0.3	2 ± 0.6	1.6 ± 0.4	1.6 ± 0.4	2.1 ± 0.3
Drylean%	26.1 ± 0.9	28.0 ±	24.9 ± 0.3	24.7 ± 0.2	26.0 ± 0.7	25.2 ± 0.4	27.9 ± 2.1	24.6 ± 0.3	25.0 ± 0.2	24.5 ± 0.5
Water%	66.8 ± 1.4	69.8 ±	68.8 ± 1.4	68.4 ± 0.7	67.7 ± 0.6	67.2 ± 0.8	67.6 ± 1.0	69.2 ± 1.2	69.4 ± 1.1	69.3 ± 2.0
Fat%	7.1 ± 1.6	2.2 ±	6.3 ± 1.6	6.8 ± 0.8	6.3 ± 1.1	7.6 ± 1.1	4.5 ± 1.2	6.2 ± 1.4	5.5 ± 1.1	6.2 ± 1.6
FEMALE										
BW	31.7 ± 1.2	33.8 ± 3.3	24.3 ± 2.3**	28.2 ± 0.6	24.8 ± 1.3	27.1 ± 0.8	25.8 ± 0.3	27.5 ± 0.6	28.7 ± 1.1	27.1 ± 1.4
Length	9.2 ± 0.1	9.5 ± 0.3	8.7 ± 0.2*	9 ± 0.2	8.8 ± 0.2	9.1 ± 0.3	8.7 ± 0.1	8.9 ± 0.1	8.7 ± 0.2	9 ± 0.2
Drymass	9.4 ± 0.6	9.8 ± 1.6	7.1 ± 0.8*	8 ± 0.5	6.3 ± 0.4	7.7 ± 0.6	6.8 ± 0.3	6.6 ± 0.2	8 ± 0.7	7.3 ± 0.5
Drylean	6 ± 0.2	6.2 ± 0.3	5.1 ± 0.4	5.9 ± 0.2	5 ± 0.2	5.4 ± 0.1	5.2 ± 0.1	5.4 ± 0.1	6 ± 0.1	5.8 ± 0.3
Water	15.7 ± 0.5	16.8 ± 0.8	14.4 ± 1.4	16.2 ± 0.2	13.3 ± 0.7	15.3 ± 0.3	14.6 ± 0.2	14.5 ± 0.3	16.4 ± 0.4*	16.5 ± 1.0*
Fat	3.5 ± 0.5	3.6 ± 1.3	2 ± 0.5	2.2 ± 0.3	1.3 ± 0.3	2.3 ± 0.5	1.6 ± 0.3	1.2 ± 0.1	2 ± 0.6	1.5 ± 0.2
Drylean%	23.7 ± 0.4	23.4 ± 0.9	24.4 ± 0.5	24.9 ± 0.3	25.6 ± 0.7	23.7 ± 0.3	24.2 ± 1.6	25.8 ± 0.3	24.8 ± 0.8	24.2 ± 0.3
Water%	62.7 ± 0.8	63.9 ± 2.8	68.2 ± 1.1	69.5 ± 0.6	68.0 ± 0.9	66.7 ± 1.6	67.1 ± 1.4	68.7 ± 0.7	67.3 ± 1.4	66.8 ± 1.2
Fat%	13.6 ± 1.1	12.7 ± 3.6	7.4 ± 1.6	7.7 ± 1.0	6.3 ± 1.2	9.6 ± 1.9	8.7 ± 1.5	5.6 ± 0.6	8.0 ± 2.1	9.0 ± 1.1

* denotes significant difference from the own (*, $p<0.05$; **, $p<0.01$).

Table 9B. Body composition of mice selectively bred for high voluntary wheel running behavior (lines 7 and 8) and the control line raised by mothers they were born to ("own"), or when they were fostered by other mothers of the same line (xf), or by other mothers of other lines (xf2, xf7, or xf8). Males, females and LF- (A), HF diet (B) are shown in separate panels.

B	own	xf	7	8	own	xf	2	own	xf	2
	2HF offspring				7HF offspring			8HF offspring		
MALE										
BW	36.6 ± 0.9	39.2 ± 1.4	35.4 ± 2.8	35.9 ± 0.7	33.7 ± 1.4	33.2 ± 0.4	34.9 ± 2.7	34.9 ± 0.9	39.4 ± 2.9	37.2 ± 0.7
Length	9.5 ± 0.1	9.6 ± 0.3	9.6 ± 0.2	9.3 ± 0.4	9.2 ± 0.2	9.5 ± 0.3	9.7 ± 0.3	9.2 ± 0.2	9.5 ± 0.0	9.7 ± 0.2
Drymass	8.9 ± 0.4	11.5 ± 0.3	9.5 ± 0.5	9.5 ± 0.5	8.6 ± 0.5	8.3 ± 0.4	9.5 ± 0.7	8 ± 0.3	10.8 ± 1.7	9.8 ± 0.6
Drylean	7.4 ± 0.2	8.5 ± 0.4	7.7 ± 0.5	7.6 ± 0.3	6.7 ± 0.2	7 ± 0.0	7.7 ± 0.5	6.9 ± 0.2	7.8 ± 0.4	7.9 ± 0.2
Water	20 ± 0.4	22.5 ± 1.2	20.8 ± 1.9	21.2 ± 0.3	18.2 ± 0.8	20.3 ± 0.1	21 ± 1.8	19.7 ± 0.5	23 ± 0.5	22.1 ± 0.5
Fat	1.5 ± 0.3	3.1 ± 0.4	1.8 ± 0.4	1.9 ± 0.3	1.9 ± 0.4	1.3 ± 0.4	1.8 ± 0.2	1 ± 0.1	3 ± 1.4	1.9 ± 0.5
Drylean%	25.7 ± 0.2	24.5 ± 0.3	25.3 ± 0.5	24.9 ± 0.2	25.2 ± 0.4	24.5 ± 0.2	25.4 ± 0.3	25.1 ± 0.2	23.3 ± 0.4	24.9 ± 0.3
Water%	69.2 ± 0.7	67.4 ± 0.5	68.9 ± 0.3	69.3 ± 1.4	68.0 ± 1.0	71.0 ± 1.2	68.4 ± 1.4	71.3 ± 0.5	68.2 ± 3.1	69.0 ± 1.0
Fat%	5.1 ± 0.8	8.1 ± 0.7	5.8 ± 0.3	5.8 ± 1.6	6.8 ± 1.2	4.6 ± 1.4	6.2 ± 1.4	3.6 ± 0.5	8.5 ± 3.5	6.1 ± 0.8
FEMALE										
BW	31.6 ± 2.0	28.6 ± 1.7	27.4 ± 3.1	31.8 ± 3.2	27.2 ± 0.4	26.8 ± 0.7	29 ± 1.0	29.6 ± 1.3	26.8 ± 0.7	28.1 ± 1.2*
Length	9.1 ± 0.2	9.1 ± 0.3	9 ± 0.4	9.2 ± 0.3	8.8 ± 0.1	8.9 ± 0.2	9.2 ± 0.2	8.9 ± 0.2	8.5 ± 0.1	9 ± 0.4
Drymass	8.9 ± 0.9	8.4 ± 0.3	8.4 ± 1.2	9.7 ± 1.4	6.9 ± 0.3	7.2 ± 0.2	7.9 ± 0.7	7.3 ± 0.8	6.7 ± 0.0	6.9 ± 0.4
Drylean	5.7 ± 0.4	5.9 ± 0.5	5.5 ± 0.5	6.2 ± 0.5	5.2 ± 0.0	5.8 ± 0.2	5.9 ± 0.3*	5.7 ± 0.2	5.9 ± 0.1	5.8 ± 0.2
Water	15.5 ± 0.8	16.2 ± 1.5	14.7 ± 1.6	17.3 ± 1.7	14.3 ± 0.2	15.5 ± 0.4	16.2 ± 0.5*	15.7 ± 0.4	15.7 ± 0.3	16.2 ± 0.8*
Fat	3.2 ± 0.6	2.5 ± 0.7	2.9 ± 0.7	3.5 ± 0.8	1.7 ± 0.3	1.4 ± 0.1	2 ± 0.4	1.6 ± 0.7	0.9 ± 0.1	1 ± 0.2
Drylean%	23.6 ± 0.5	23.8 ± 0.8	24.7 ± 0.3	25.2 ± 0.3	24.7 ± 0.3	25.4 ± 0.3	24.2 ± 0.8	24.7 ± 0.6	26.1 ± 0.2	23.2 ± 0.6
Water%	63.8 ± 1.3	65.6 ± 2.6	67.2 ± 1.8	70.3 ± 0.1	67.4 ± 1.2	68.2 ± 0.3	64.0 ± 1.4	68.6 ± 1.7	70.1 ± 0.5	64.2 ± 1.1
Fat%	12.7 ± 1.7	10.6 ± 3.3	8.1 ± 1.5	4.5 ± 0.5	7.9 ± 1.4	6.4 ± 0.3	11.8 ± 2.2	6.7 ± 2.2	3.8 ± 0.7	12.5 ± 1.6

* denotes significant difference from the own (*, $p<0.05$; **, $p<0.01$).

6.5. Hormone levels of cross-fostered offspring

In the LF condition, fostering of 2LF offspring by 8LF mothers caused significant effects on plasma levels of insulin ($F(3,30)=3.03$; $p<0.05$) and glucose ($F(3,30)=6.34$; $p<0.01$). See Table 10. Specifically, 2LF offspring had lower levels of insulin when they were fostered by 8LF mothers and in fact were indistinguishable from 8LF own offspring. Glucose levels were also decreased when 2LF offspring were fostered by 7LF and 8LF mothers, and were indistinguishable from to 8LF own offspring and even lower than in 7LF own offspring. No effects on plasma levels of adiponectin and leptin were observed by cross-fostering. Cross-fostering of pups between mothers in the same line caused an increased in insulin levels in 8HF offspring ($p<0.05$), but no other effects were observed.

6.6. Food and water intake of cross-fostered offspring

Food and water intake assessed at 3 months of age are shown in Table 11. No effect of cross-fostering within or between lines was found on food and water intake.

6.7. Running wheel behavior of cross-fostered offspring

Running wheel behavior was assessed according to the same methodology as used for selection of lines. Therefore the average of the 5th and 6th day of total number of wheel revolutions was analysed and since rodents are active in the dark phase, the dark phase of the 6th day was also examined specifically. No effect of cross-fostering was found in any line and diet condition. See Table 12.

6.8. Plus maze performance of cross-fostered offspring

Line 2LF offspring fostered by line 8LF mothers showed an interaction effects with gender on time spent in open arms ($F(2,25)=9.25$; $p<0.001$), %open arm entries ($F(2,25)=5.65$; $p<0.01$), closed arm entries ($F(2,25)=7.25$; $p<0.005$) and %closed arm entries ($F(2,25)=5.65$; $p<0.01$) (Table 13). Specifically, female offspring of 2LF fostered by 8LF mothers spent more time in open arms, had higher percentage open arm entries, less closed arm entries and lower percentage closed arm entries than 2LF own offspring. Line 7LF offspring fostered by 2LF mothers showed an interaction with gender on time spent in closed arms ($F(1,22)=5.05$; $p<0.05$) and time spent in the center ($F(1,22)=4.60$; $p<0.05$). Specifically, male offspring of 7LF spent more time and females spent less time in the closed arms. Male, but not female, line 7LF offspring spent less time in the center when they were fostered by 2LF mothers.

In the HF diet condition, 2HF offspring during plus maze test had only one male and one female mice in the group when they were cross-fostered to 8HF mothers, therefore, results were not shown. No effects were observed in 7HF and 8HF offspring.

Table 10. Plasma hormone and glucose levels of mice selectively bred for high voluntary wheel running behavior (lines 7 and 8) and the control line raised by mothers they were born to ("own"), or when they were fostered by other mothers of the same line (xf), or by other mothers of other lines (xf2, xf7, or xf8). LF-, HF diet are shown in separate panels.

	own	xf	7	8	own	xf	2	own	xf	2
	2LF offspring				7LF offspring			8LF offspring		
MALE										
glucose (mM)	9.4 ± 0.9	5.7 ±	6.4 ± 0.8	6.2 ± 0.8	8.5 ± 0.6	8 ± 0.4	8.9 ± 0.8	6.9 ± 0.9	9.2 ± 1.2	9 ± 0.3
insulin (ng/ml)	1.7 ± 0.5	1.2 ±	0.6 ± 0.1	0.9 ± 0.7	1.2 ± 0.2	0.8 ± 0.3	0.9 ± 0.5	0.6 ± 0.1	0.7 ± 0.3	1.5 ± 0.5
leptin (ng/ml)	2.5 ± 0.6	1.3 ±	1.3 ± 0.1	1.5 ± 0.2	3 ± 0.4	2.9 ± 0.6	2.4 ± 0.7	1.8 ± 0.3	1.8 ± 0.3	2.4 ± 0.6
adiponectin (µg/ml)	3.8 ± 0.6	2 ±	2.5 ± 0.2	2.7 ± 1.2	5.2 ± 0.4	6 ± 0.2	4 ± 0.6	4.3 ± 0.6	4.1 ± 0.6	5 ± 0.6
FEMALE										
glucose (mM)	9.5 ± 0.8	8 ± 0.8	6.2 ± 0.8*	6.9 ± 0.8	7.4 ± 0.4	6.6 ± 0.4	5.9 ± 0.5	7.1 ± 0.4	7.6 ± 0.7	6.2 ± 0.3
insulin (ng/ml)	1.5 ± 0.4	1.5 ± 0.6	0.7 ± 0.2	0.1 ± 0.1*	0.4 ± 0.2	0.6 ± 0.3	0.2 ± 0.1	0.6 ± 0.2	0.8 ± 0.3	0.2 ± 0.1
leptin (ng/ml)	5.4 ± 1.0	7.1 ± 2.0	2.5 ± 0.7	2.5 ± 1.0	2.6 ± 0.6	3.5 ± 0.9	1.6 ± 0.2	1.6 ± 0.2	2.4 ± 0.8	1.3 ± 0.5
adiponectin (µg/ml)	8.3 ± 0.5	9 ± 1.1	6.9 ± 0.7	8.5 ± 2.1	9.6 ± 0.8	8.5 ± 0.8	9.5 ± 2.0	11.2 ± 0.7	9.1 ± 1.8	9.5 ± 0.8
	2HF offspring				7HF offspring			8HF offspring		
MALE										
glucose (mM)	7.8 ± 0.5	7.6 ± 1.0	7.6 ± 1.3	9.5 ± 1.6	7.7 ± 1.0	7.1 ± 0.5	7.2 ± 0.0	7.9 ± 0.6	9.7 ± 1.8	9.2 ± 2.0
insulin (ng/ml)	0.8 ± 0.3	1.3 ± 0.2	1.3 ± 0.7	1 ± 0.3	1 ± 0.2	0.8 ± 0.1	1.4 ± 1.0	0.5 ± 0.1	1.2 ± 0.1*	0.6 ± 0.4
leptin (ng/ml)	2.3 ± 0.5	3.2 ± 0.7	2.5 ± 0.6	1.8 ± 0.5	2.6 ± 0.6	1.6 ± 0.0	1.3 ± 0.6	1.2 ± 0.2	2.2 ± 0.9	1.6 ± 0.6
adiponectin (µg/ml)	4.5 ± 1.1	3.5 ± 0.5	4 ± 0.9	3.6 ± 0.5	4.7 ± 0.3	5.4 ± 1.4	4.6 ± 0.7	4.3 ± 0.5	3.7 ± 0.7	4.7 ± 1.2
FEMALE										
glucose (mM)	7.5 ± 0.6	7.3 ± 0.9	7.5 ± 0.6	8.5 ± 0.7	7.1 ± 0.8	5.9 ± 0.9	8.3 ± 0.9	7.4 ± 0.7	7.5 ± 1.3	8.3 ± 0.2
insulin (ng/ml)	0.7 ± 0.3	0.8 ± 0.5	1.2 ± 0.2	1 ± 0.2	0.3 ± 0.1	0.5 ± 0.2	0.9 ± 0.6	0.3 ± 0.2	0.9 ± 0.6	0.2 ± 0.2
leptin (ng/ml)	4.3 ± 0.8	3.1 ± 1.1	3.5 ± 0.9	4.2 ± 0.4	2.2 ± 0.3	1.7 ± 0.1	3.3 ± 1.2	1.9 ± 0.7	1.7 ± 0.2	1.3 ± 0.1
adiponectin (µg/ml)	6.9 ± 1.0	7.2 ± 0.5	10 ± 3.9	7.2 ± 1.2	10 ± 0.8	8.8 ± 0.7	10.8 ± 1.1	12.7 ± 0.9	9.1 ± 0.5	11.3 ± 1.4

* denotes significant difference from the own (*, $p<0.05$).

Table 11. Food and water intake of mice selectively bred for high voluntary wheel running behavior (lines 7 and 8) and the control line raised by mothers they were born to ("own"), or when they were fostered by other mothers of the same line (xf), or by other mothers of other lines (xf2, xf7, or xf8). LF-, HF diet are shown in separate panels.

	own	xf	7	8	own	xf	2	own	xf	2
	2LF offspring				7LF offspring			8LF offspring		
MALE										
Food intake (g)	10.5 ± 0.7	13.7 ±	11.7 ± 1.1	11.5 ± 0.2	13.4 ± 0.4	13.5 ± 0.3	12.2 ± 0.4	14.2 ± 1.7	12 ± 0.6	13.2 ± 0.4
Water intake (g)	11 ± 1.0	12.6 ±	10.8 ± 0.5	11.1 ± 0.3	12.7 ± 1.4	13.2 ± 0.5	13.1 ± 1.2	12.8 ± 0.9	8.7 ± 1.6	14.4 ± 2.9
FEMALE										
Food intake (g)	9.1 ± 0.4	10.83 ± 1.13	7.9 ± 0.6	8.9 ± 1.3	14.3 ± 0.7	12.8 ± 1.0	15 ± 1.3	11 ± 0.5	12.1 ± 1.3	11.1 ± 1.3
Water intake (g)	9.4 ± 0.5	8.3 ± 1.7	9.9 ± 0.7	10.3 ± 1.0	16.7 ± 1.0	14.4 ± 1.8	14.9 ± 0.8	12 ± 1.3	13.7 ± 0.6	11 ± 1.0
	2HF offspring				7HF offspring			8HF offspring		
MALE										
Food intake (g)	15.9 ± 2.6	11.4 ± 0.7	12 ± 0.9	9.8 ± 0.2	14.2 ± 1.0	16.4 ± 1.0	16 ± 1.0	17.4 ± 1.9	13.7 ± 0.5	12.6 ± 0.1
Water intake (g)	14.1 ± 1.3	11.1 ± 0.6	12.8 ± 1.2	5.5 ± 3.4	14.4 ± 0.7	17.4 ± 1.2	16.2 ± 0.5	17.2 ± 1.1	15.2 ± 1.2	13.3 ± 3.0
FEMALE										
Food intake (g)	8.1 ± 0.8	9.6 ± 2.1	13.1 ± 2.4	8.2 ± 1.9	12.4 ± 1.5	13.9 ± 0.6	14.5 ± 2.0	14.7 ± 1.6	14.3 ± 1.8	14.4 ± 0.6
Water intake (g)	9.5 ± 0.6	8.6 ± 1.3	9.8 ± 0.7	16.1 ± 6.3	13.9 ± 2.7	15.7 ± 1.6	21.1 ± 3.6	13.4 ± 1.0	13.4 ± 0.4	15.5 ± 0.8

No significant effect was found.

Table 12. Running wheel activity of mice selectively bred for high voluntary wheel running behavior (lines 7 and 8) and the control line raised by mothers they were born to ("own"), or when they were fostered by other mothers of the same line (xf), or by other mothers of other lines (xf2, xf7, or xf8). LF-, HF diet are shown in separate panels.

	own	xf	7	8	own	xf	2	own	xf	2
	2LF offspring				7LF offspring			8LF offspring		
MALE revolutions/day	18171 ± 5637	6403 ±	6662 ± 352	7924 ± 436	14443 ± 2031	27277 ± 1433	19279 ± 3322	21983 ± 2881	36462 ± 3798	22409 ± 4030
FEMALE revolutions/day	11603 ± 3935	20040 ± 2430	14342 ± 1753	14751 ± 3890	37103 ± 5363	48710 ± 28058	24736 ± 6451	53909 ± 10309	19623 ± 9161	31072 ± 5999
	2HF offspring				7HF offspring			8HF offspring		
MALE revolutions/day	18189 ± 7043	18766 ± 3494	7545 ± 1754	30448 ± 11447	22575 ± 7928	18088 ± 346	17716 ± 3723	25974 ± 4113	44616 ± 8627	16291 ± 7198
FEMALE revolutions/day	16253 ± 1323	17072 ± 10131	20784 ± 4907	17895 ± 3862	34784 ± 5928	28696 ± 4541	30822 ± 9677	38172 ± 18817	37292 ± 12674	39446 ± 8158

No significant effect was found.

Table 13. Plus maze performance behavior of mice selectively bred for high voluntary wheel running behavior (lines 7 and 8) and the control line raised by mothers they were born to ("own"), or when they were fostered by mothers of other lines (xf2, xf7, or xf8). Males, females and LF-, HF diet are shown in separate panels.

	own	7	8	own	2	own	2
	2LF offspring			7LF offspring		8LF offspring	
MALE							
%Topen	13.6 ± 3.6	6.4 ± 4.1	10.5 ± 5.9	4.2 ± 2.5	2.7 ± 1.5	8.6 ± 2.7	10.2 ± 5.1
%Tclosed	40.2 ±3.6	50.4 ± 3.9	53.8 ± 4.6	40.8 ± 4.6	61.7 ± 9.2*	62.6 ± 19.8	58.5 ± 8.2
%Tcenter	46.2 ± 2.4	43.2 ± 1.5	35.7 ± 4.6	55 ± 3.9	35.6 ± 8.0*	28.9 ± 9.1	31.3 ± 4.8
open entries	4 ± 1.0	3.7 ± 2.0	4 ± 2.6	2.3 ± 1.4	2 ± 1.4	2.7 ± 0.9	3.7 ± 1.6
closed entries	14.1 ± 1.5	11.7 ± 0.3	18.7 ± 4.3	19.9 ± 1.9	15.8 ± 2.6	12.9 ± 4.1	14.2 ± 1.1
total entries	18.1 ± 1.9	15.3 ± 2.3	22.7 ± 3.4	22.2 ± 1.7	17.8 ± 3.5	15.6 ± 4.9	17.8 ± 1.9
%open entries	21.4 ± 5.1	20.6 ± 10.9	18.3 ± 10.4	9.91 ± 5.4	8.9 ± 5.2	16 ± 5.1	18.3 ± 6.6
%close entries	78.6 ± 5.1	79.4 ± 10.9	81.8 ± 10.4	90.1 ± 5.4	91.1 ± 5.2	84 ± 26.6	81.7 ± 6.6
FEMALE							
%Topen	8.5 ± 3.0	12.2 ± 2.9	57.2 ± 19.2**	10.2 ± 2.6	12.4 ± 7.6	13.6 ± 2.2	12.8 ± 5.5
%Tclosed	51.4 ± 5.4	56 ± 5.4	15.3 ± 8.5**	45 ± 3.9	39.2 ± 7.6	53.8 ± 2.4	48 ± 5.9
%Tcenter	40.2 ± 3.8	31.8 ± 4.1	27.5 ± 11.0	44.8 ± 4.7	48.5 ± 2.8	32.6 ± 2.4	39.2 ± 4.3
open entries	3.4 ± 0.7	6.7 ± 1.9	6 ± 2.9	5.2 ± 1.3	4.3 ± 1.3	4.7 ± 0.9	2.3 ± 1.3
closed entries	15.3 ± 0.9	14.5 ± 1.3	6.3 ± 3.8**	18.2 ± 1.9	17.5 ± 3.9	15.8 ± 1.2	14.8 ± 1.1
total entries	18.6 ± 1.3	21.2 ± 2.4	12.3 ± 6.6	23.4 ± 2.9	21.8 ± 3.6	20.5 ± 1.2	17 ± 1.2
%open entries	17.1 ± 3.1	29.4 ± 6.3	65.3 ± 17.4**	20.1 ± 3.9	21.6 ± 8.2	22.8 ± 3.7	12.5 ± 6.5
%close entries	82.9 ± 3.1	70.6 ± 6.3	34.7 ± 17.4**	79.9 ± 3.9	78.4 ± 8.2	77.2 ± 3.7	87.5 ± 6.5
	2HF offspring			7HF offspring		8HF offspring	
MALE							
%Topen	26.9 ± 12.9	6.2 ± 1.9	29.3 ±	16.7 ± 6.0	8.5 ± 5.3	15.3 ± 1.8	11.6 ± 2.2
%Tclosed	49.4 ± 11.8	56.4 ± 8.8	6.6 ±	51.3 ± 8.0	58.6 ± 13.8	50.1 ± 4.0	62.7 ± 10.6
%Tcenter	23.7 ± 3.0	37.4 ± 9.1	64.1 ±	32.1 ± 9.3	32.9 ± 8.5	34.7 ± 3.6	25.7 ± 9.2
open entries	3.6 ± 1.3	4 ± 0.0	5 ±	7.7 ± 2.1	5 ± 2.5	4.3 ± 1.0	3.7 ± 1.5
closed entries	9.2 ± 2.3	12.7 ± 2.3	2 ±	12.5 ± 1.7	10 ± 2.5	14.3 ± 1.5	10.7 ± 3.2
total entries	12.8 ± 1.5	16.7 ± 2.3	7 ±	20.2 ± 3.7	15 ± 4.0	18.5 ± 1.8	14.3 ± 4.6
%open entries	31.9 ± 12.4	24.9 ± 3.4	71.4 ±	34.4 ± 4.1	32.1 ± 9.7	22.6 ± 3.5	23.5 ± 3.4
%close entries	68.1 ± 12.4	75.1 ± 3.4	28.6 ±	65.6 ± 4.1	67.9 ± 9.7	77.4 ± 3.5	76.5 ± 3.4
FEMALE							
%Topen	7.6 ± 4.4	25.5 ± 12.8	0.7 ±	16.7 ± 6.9	15.5 ± 8.0	13.1 ± 2.8	17.2 ± 3.0
%Tclosed	35 ± 5.2	42.9 ± 3.4	70.3 ±	51.7 ± 5.8	49.9 ± 8.1	52.8 ± 3.4	48.7 ± 5.1
%Tcenter	57.4 ± 6.2	31.6 ± 10.4	29 ±	31.6 ± 1.5	34.6 ± 0.1	34.2 ± 4.4	34.1 ± 3.7
open entries	3.3 ± 1.6	4 ± 2.3	1 ±	7.6 ± 2.2	6.7 ± 4.1	4.8 ± 0.7	5.3 ± 1.9
closed entries	15.8 ± 2.8	12.3 ± 2.6	18 ±	19.6 ± 2.1	18 ± 2.5	16.5 ± 1.3	15.7 ± 1.2
total entries	19 ± 2.7	16.3 ± 2.3	19 ±	27.1 ± 2.7	24.7 ± 4.7	21.3 ± 1.1	21 ± 3.0
%open entries	16.7 ± 7.8	24.4 ± 12.4	5.3 ±	26.3 ± 7.8	24.3 ± 12.4	23 ± 3.9	24.1 ± 4.9
%close entries	83.3 ± 7.8	75.6 ± 12.4	94.7 ±	73.7 ± 7.8	75.7 ± 12.4	77 ± 3.9	75.9 ± 4.9

* denotes significant difference from the own (*, $p<0.05$; **, $p<0.01$).

7. Discussion on perinatal nutritional effects of cross-fostered offspring

Mice selectively bred for increased running wheel behavior have several behavioral, physiological, and metabolic changes compared to non-selected mice, among which some may be viewed as co-adaptations to sustain endurance exercise (Girard et al. 2001; Gomes et al. 2009; Houle-Leroy et

al. 2003; Koteja et al. 1999; Vaanholt et al. 2007; Wong et al. 2009; Rezende et al. 2006a) and also (Chapter 2, 3, 4 of this thesis). We were interested to know to which extent the post-natal phase is critically important for the display of these phenomena. During the post-natal period, maternal influences are reported to cause programming effects in the developing offspring and subsequently cause long-lasting changes at adulthood (Armitage et al. 2005; Gallou-Kabani et al. 2007; Napoli and Palinski 2001; Samuelsson et al. 2008). For this reason, we performed an experiment in which we cross-fostered pups between mothers from different lines and between mothers from the same line. These comparisons were made when mothers were either feeding a low-fat (LF) carbohydrate-rich diet, or a high- fat (HF) diet enriched with refined sugars. Offspring growth during lactation and several parameters relevant to energy balance regulation after weaning were assessed in these cross-fostered mice, and were compared to the characteristics of the non cross-fostered offspring described in the first part of this chapter.

It was previously mentioned that growth rates of mice from the high activity lines 7 and 8 are lower than those of the control line 2 during the pre-weaning period as well as during adulthood. These effects were interpreted to indicate that selection for running wheel behavior causes a reduction in body size and mass which may be adaptive to sustain running wheel behavior (Rezende et al. 2006b). Cross-fostering of pups between line 2 and line 8 mothers feeding the LF diet (further referred to as 2LF and 8LF mothers) diet did not show alterations in growth during the pre-weaning stage compared to the line 2LF and 8LF pups raised by their own mothers. This indicates that differences between line 2 and 8 offspring are probably the result of differences in pre-natal (epi) genetic programming, and not by post-natal influences. In contrast, line 7LF offspring fostered by line 2LF mothers increased pup growth of line 7LF pups in the direction of 2LF own pups and became indistinguishable from cross-fostered 8LF pups. Vice versa, line 2LF offspring fostered by line 7LF mothers showed a markedly attenuated growth, even below the levels found in the line 7LF offspring. Therefore line 7 mothers provide a poor post-natal environment in the LF diet condition for offspring growth and development, and essentially similar effects are observed when mothers were feeding a HF diet. In the HF diet condition, both line 7 as well as line 8 offspring fostered by line 2HF mothers increased pup growth compared to condition when they were raised by their own mothers, thus presenting a richer environment for growth and development in line 2 mothers relative to mothers in the high activity lines, when the mothers are provided with an energy rich diet.

Effects of the poor pre-weaning maternal line 7 environment persistently reduced body weight of line 2 offspring in adulthood. In fact, the line 2 offspring at 4 months of age fostered by line 7 mothers were indistinguishable from line 7 own offspring in terms of dry-lean mass, fat mass, and body length. On the others hand, line 7LF offspring fostered by line 2LF mothers were only slightly and transiently heavier than line 7 offspring raised by their own LF mothers. These effects rely on the type of diet, because all cross-fostering effects are lost in adulthood when mothers are feeding a HF diet. Two conclusions can be drawn from these findings. First, the poor maternal line 7LF environment causes long-lasting physiological and morphological

changes that overrule pre-natal (epi)genetic make-up of line 2 offspring, and may potentially contribute to some extent to the phenotype of line 7 mice at adulthood as well. Secondly, the pre-natal (epi)genetic make-up of line 7 offspring cannot be overridden by the relatively rich post-natal environment provided by line 2 mothers, even when they are subjected to a HF diet. A poor environment may therefore have stronger effects on long-term programming of energy balance and growth than a rich environment. While this is consistent with the current conceptual framework of developmental metabolic programming founded on the idea of the thrifty phenotype and the pioneering work of Hales and Barker (Hales and Barker 1992), the directions of body weight changes in the current study are not consistent with this idea. According to the thrifty phenotype hypothesis, poor postnatal nutrition would be expected to cause catch-up growth (Gluckman and Hanson 2004; Gluckman, Hanson, and Pinal 2005), and in line with that we anticipated that line 2 offspring fostered by line 7 mothers would develop obesity and hyperglycemia at adulthood. In stead, they developed lower growth and lower growth efficiency (based on lower body weights combined with similar food intake), and lower levels of glucose and insulin at adulthood. The observed long-term fostering effects of line 7 mothers are not generalizable to selection for increased running wheel behavior per se, since cross-fostering of line 2 and line 8 pups by respectively line 8 and line 2 mothers did not yield aforementioned effects on adult body weights. In this case, differences in body weight and composition between these lines are apparently determined by prenatal gene-environment interactions. Neither did line 7 nor line 8 cross-fostering with line 2 result in changes in running wheel behavior, food intake nor water intake. The only effect of cross-fostering which we observed was that line 7 female offspring fostered by line 2 mothers became more anxious in the plus maze performance test, whereas line 2 female offspring fostered by line 8 mothers became less anxious. This indicates some postnatal effects of line 2 mothers to increase anxiety levels in their female offspring which might be offset in the high activity lines.

A remarkable observation in Chapter 5 was that line 7 mothers lost considerably more pups during lactation than mothers of other lines. Although litter characteristics at birth in Chapter 5 were very similar to those in this study, here we did not find pups missing during lactation. We suspect that these differences are the result of the cross-fostering procedure in the present study. Surviving the post-natal environment is not only dependent on maternal (line) and dietary (HF/LF) influences acting unidirectional on the offspring. For example, suckling (Schroeder et al. 2007; Fride, Bregman, and Kirkham 2005; Uvnas-Moberg 1996) and ultrasonic vocalization by pups (Shair 2007; Hofer et al. 1999) have been reported to markedly affect maternal behavior and physiology, and this in turn feeds back to pup growth and development. It may be speculated, that the pup losses of line 7 mothers that we have observed in Chapter 5 were "rescued" by line 2 pups fostered by line 7 mothers in the present study. A mechanism may be that line 2 pups suckled more vigorously than line 7 pups, and thus contributed to a higher milk production of line 7 mothers in the present study, benefiting both fostered and own offspring. Along these lines, also differences in growth efficiency (GE) during lactation found in

the present study and Chapter 5 may be explained. Thus, while line 7HF and 8HF mothers and offspring had the highest GEs in Chapter 5, here we observed that GE of line 2HF mothers was the highest. These effects may very well be explained by the contribution of growth efficiency of line 7HF and line 8HF offspring which were fostered by line 2 HF mothers. Further studies are needed to further explore these potential mechanisms, and whether they are relevant to behavioral and metabolic characteristics at adulthood.

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CHAPTER 8

GENERAL DISCUSSION:

EFFECTS OF "NATURE" AND "NURTURE" IN VOLUNTARY ACTIVITY AND NUTRITION ON ENERGY BALANCE AND EMOTIONALITY

1. Introduction

Animals are able to travel across the environment in the attempt to overcome environmental challenges (e.g., escaping predation, climate change, etc.), or to achieve certain goals (e.g., find nutrients, fluids, partners, social interaction, etc.). In doing so, they need to combust extra fuels above the basic requirements, in order to provide energy for skeletal muscular and cardiovascular activity. The level of locomotor activity can vary tremendously between species, but also considerable individual differences can be observed within certain species. An increased level of locomotor activity may increase survival rate of animals by allowing the coverage of larger habitats. Co-adaptational changes may be needed for animal physiology, morphology and behavior to facilitate and sustain high levels of physical activity. Selective breeding for voluntary physical activity is an appropriate tool to study potential co-adaptational changes, since selective breeding for one trait could be linked to changes of several sub-ordinate traits that may determine individual variation. In this thesis, I used two lines of mice selectively bred for increased wheel running behavior and one control line created by Prof T. Garland and colleagues. The aim was to investigate in these lines of mice regulation of energy homeostasis under non-reproductive and reproductive conditions, and its contribution to perinatal programming of energy homeostasis in the offspring. Below, the main results from the different studies are summarized.

The aim of **Chapter 2** was to study behavioral consequences of selective breeding for high voluntary activity. The results demonstrated that mice with high activity levels responded differently in novel and habituated environments. In a novel environment, high activity mice were more anxious, more explorative and risk-taking suggesting that their level of carefulness, cautiousness and attentiveness was increased relative to non-selected mice. In a habituated environment, they had increased routine behavior, which probably helps them to sustain their physical endurance activity.

In **Chapter 3**, activity selected and control mice were subjected to feeding either a carbohydrate-rich/low-fat (LF) or a 60% high-fat (HF) diet and temporal changes in energy balance characteristics over the daily cycle were studied. Compared to controls, physically active mice had an increased locomotor activity and increased energy expenditure (particularly in the HF diet condition) during the dark phase. In active males, the latter was particularly due to increased carbohydrate oxidation. In contrast, active females showed a strongly increased fat oxidation, particularly on the HF diet. Since active females started ingesting their bulk food later in their active phase, this might make them more prone to metabolic insufficiency. In view of their leanness, their LF diet preference over the HF diet was surprising, but may be viewed as a result of metabolic sensors potentially signaling shortage of intermediary citric acid metabolites necessary for fat oxidation, and refers to old adage that "fats burn in the flame of carbohydrates".

In **Chapter 4**, control and activity selected mice were subjected to a LF or a 40% HF diet with added refined sugars, and the effects were studied on parameters related to energy balance and emotional stress. The results showed that the active lines were HF diet obesity resistant, with increased food intake, similar absorption rates and higher non-exercise

thermogenesis (NEAT) than their randomly bred controls. While activity selected females had a higher anxiety level in the LF condition than controls, this effect reversed on a HF diet, without concomitant changes in plasma levels of corticosterone. Since active lines again had a lower HF preference than controls, these data may be viewed such that the activity lines, and in particular the females may improve their mood on a HF diet, without necessarily liking the HF diet more than control mice.

In **Chapter 5**, the differences between control and activity selected mice were investigated on reproductive performance during the perigestational period in relation to feeding a LF or 40% HF diet with added refined sugars. The results showed that the active lines had increased reproduction output and an increase in growth efficiency during peak lactation on the HF diet relative to controls, but had mixed advantageous effects on pup survival. Regression analysis revealed that litter size acted as a strong dependent variable, and effects of diet and line on several pup characteristics remained significant within the regression analysis.

In **Chapter 6**, we aimed to study the long-term consequences of feeding HF or LF diets during the perigestational period in activity-selected mice and in their controls on energy balance regulation in the offspring. The outcomes showed that active lines are protected against the early programming effects of HF diet feeding as demonstrated by the maintenance of a lean phenotype, elevated plasma levels plasma of adiponectin, and low plasma levels of leptin and insulin. In contrast, their randomly bred controls had increased longitudinal growth, higher adipose tissue mass, and elevated insulin and low adiponectin levels. Again, regression analysis revealed that litter size the animals were born in was an important dependent factor, but with diet and line having particular effects on structural growth parameters.

Chapter 7 had two aims: A) to study the contribution of perigestational HF or LF diet feeding in activity selected mice and in controls independent of differences in litter size on reproductive performance and offspring characteristics and B) to study postnatal influences in active and control mice by cross-fostering of pups between lines. In part A) the results demonstrated that equalizing litter size to 10 pups/litter prevented perigestational HF diet effects on postnatal body weight gain in the control line, but instead caused transiently increased weight gain and increased adiposity at the adolescent stage in the activity selected mice. An explanation for these unexpected results is that equalizing litters frequently needed culling down in the active lines, whereas those of the control line often needed additional pups. In this situation, the highly active mothers suddenly faced on average two pups less than they prepared for, and probably over-nourished their offspring to higher average body weights and adiposity. Controls on the other hand, had to nurse more pups than they prepared for, and this could have lead to a normalization of body weight and body adiposity levels. Effects of over-nourishment in the activity-selected lines, however, were transient and overridden by trait at adulthood.

In part B) cross-fostering experiments revealed that females of selection lines provided different postnatal environments to their offspring than control females, but also differences between activity selected lines 7 and 8 were observed. When pups were exchanged between the

control and selected line 8 mothers, pup growth was not affected. However, when pups were fostered between control and selected line 7 mothers, control pups became smaller and line 7 pups became transiently larger. The postnatal environment of selected lines, irrespective of diet, resulted in low plasma levels of insulin and glucose in control animals, overriding the prenatal (epi)genetic make-up of control offspring later in life. However, prenatal genetic make-up of selected lines could not be overridden at adulthood by a relatively rich environment of controls even in the presence of a HF diet during the perigestational period. Interestingly, growth efficiency during lactation of pups and mothers of the control line became increased on a HF diet. This was different compared to the results in Chapter 5, and may be caused by the contribution of fostered selected pups in the control litters. Furthermore, the pup loss of line 7 mothers found in Chapter 5 did not occur in the line 7 litters with fostered control pups of line 2 in the present study. This improved pup survival could indicate that pup loss in line 7 litters of Chapter 5 was the result of bi-directional pup mother interactions rather than the result of poor maternal care per sé.

2. Connecting physical activity and emotionality/personality

In nature, animals invading novel habitats and having no prior experiences with their challenges usually have increased stress levels since they do not know what to expect (e.g., of predators, climate etc.). However, they need to overcome these emotional challenges and find nutrients, water, partners etc, to secure their own survival as well as that of its progeny. As mentioned earlier, highly active animals might have an advantage to cover larger habitats, but exposure to the outside environment may require other specific qualities to improve survival. In the present thesis, highly active mice were found to be explorative, risk taking and at the same time had increased levels of anxiety in unfamiliar conditions, which may translate to a cautious and attentive behavior relative to that of control mice (Chapter 2). On the other hand, in a habituated environment, they were less attentive, and increased their stereotype, routine behavior. Although the high activity mice were more resistant to develop diet-induced obesity (DIO) than control mice when subjected to a HF diet (Vaanholt et al. 2008), the HF diet did appear to have a large influence on these personality traits in the activity selected animals just as well as did in the control mice. The directions of effects, however, were quite different between control and selected lines. In the control mice, for example, the HF diet increased anxiety in unfamiliar conditions, but the opposite was observed in the activity selected mice. These differential effects are reminiscent of opposing reports in literature that fats and refined sugars can either be comforting and decreasing stress sensitivity (Dallman, Pecoraro, and la Fleur 2005), or act as a form of background stress (Dallman, Pecoraro, and la Fleur 2005; Souza et al. 2007; Tannenbaum et al. 1997). The data in the presented studies may be interpreted such that (a trait for) physical activity acts as a lever to determine the direction of emotionality behavioral when subjected to diets differing in HF and sugar content. Congruent changes in neuroendocrine and

physiological parameters were less clear, although emotional and behavioral effects of HF diets have previously been shown to have specific neuroendocrine and physiological correlates (Steimer, la Fleur, and Schulz 1997). From a teleological standpoint, a too dramatic decrease in the level of anxiety may negatively affect chances of survival. However, without the beneficial effects of diet-induced thriftiness (i.e., which probably enables the control mice to survive upcoming periods of famine better than the activity selected mice), a trait for increased physical activity might benefit survival in well-fed conditions by augmenting agility, extraversion and fearlessness under unfamiliar conditions.

3. Life-style factors in relation to complex diseases

Besides understanding neurobiology of energy balance regulation from an adaptive standpoint, animal models with selected complex traits have also been used for understanding pathological mechanisms and treatment effectiveness of human diseases. Human studies suggest a complex interplay of life-style factors, including behavioral inactivity, "unhealthy" eating habits, in the aetiology and manifestation of disease. Again, stress might play an important role since, for example, the metabolic syndrome is found more prevalent among individuals under psychosocial pressure or chronic stress (Bjorntorp 1992; Brunner et al. 2002), and this has been associated with emotional eating, binge-eating and depression as well (Pinaquy et al. 2003). Furthermore, several reports taken together seem to suggest that a change in any of these life-style factors can bring about changes in the others, and ignite a chain reaction leading to pathology development (van Dijk and Buwalda 2008; Tsatsoulis and Fountoulakis 2006; Kishi and Elmquist 2005; Foreyt 2006). Different disciplines revealed that physical activity can be used as a preventive or corrective tool in many metabolic abnormalities and diseases (Hayes and Kriska 2008; Brock et al. 2005; Colberg 2007; Donnelly et al. 2009), as well as emotional and mental complications (Antunes et al. 2005; Budde et al. 2008; Galper et al. 2006; Kramer and Erickson 2007; Kruk 2007; Otto et al. 2007; Pearce 2008). Therefore, studying physiology and behavior in animals with complex traits for high physical activity can help to improve our understanding of the underlying mechanisms of these prevention and treatment options.

4. Physiological co-adaptations of endurance activity

Endurance exercise requires an increase in aerobic capacity and increased oxygen consumption, which is strongly related to an increase in heart-minute volume during activity. This facilitates oxygen perfusion of energetically demanding organs and increases transport of carbon dioxide and waste products away from these organs (Laughlin and Roseguini 2008; Betik et al. 2009; Baar 2009; Hickson, Bomze, and Holloszy 1977). To serve these energetic requirements, nutrients need to be taken up from ingested food and transported to the demanding organs or first being

stored in the form of glycogen or fat, and released later. Because of higher voluntary activity and energy expenditure in the selected mice, an increase in food intake behavior needs to be temporally spaced between bouts of activity across the circadian cycle. In the selected mice, this caused ingestive behavior at times when controls were not feeding (Chapter 3). Not only the amount but the type of ingested food is important to support energy utilization. In the present experiments, the activity selected mice had increased preference for carbohydrate-rich food over fat food, potentially to secure their fatty acid oxidation rate (chapter 3). A decrease in fat tissue and a shift to an increase in dry lean mass is associated with the increased rate of fat oxidation, and others have shown that this is paralleled by an increase in the number of mitochondria and oxidative muscle types (Guderley et al. 2006; Guderley et al. 2008; Houle-Leroy et al. 2000). Since the increased nutrient delivery during physical activity can be caused independent of insulin (Hamilton and Booth 2000), the level of the metabolic hormones insulin and leptin, which promote glucose uptake and lipid storage, are less important under conditions of chronic exercise. The high level of adiponectin, on the other hand *is* important since this hormone has been shown to stimulate mitochondrial biogenesis and to augment fatty acid oxidation in skeletal muscle (Yamauchi et al. 2002; Civitarese et al. 2006).

Over the last decades, environmental factors largely changed and favored consumption of comfort foods, containing high percentages of saturated fats and/or sugars. In order to maintain body weight and energy homeostasis, this "nutritional challenge" should be balanced by a higher intensity of physical activity, to reverse metabolic abnormalities. The mechanism by which physical activity can reverse these abnormalities is related to an increased non-exercise thermogenesis (NEAT) (chapter 4). Increasing sports during leisure time may be less effective, since this may lead to compensations by resting more (McCrady and Levine 2009; Levine 2007). A high level of NEAT, however, may clear triglycerides and increase skeletal muscle oxidative capacity as well. According to the findings in Chapter 3, this might stimulate carbohydrate preference, allowing that the dietary or stored fat is burning in the flame of carbohydrates (chapter 3).

Although leanness and increased aerobic capacity as a result of endurance training are not associated with a clear reduction in basal metabolic rate (Bouchard, Depres, and Tremblay 1993), there is however evidence that it increases metabolic efficiency in humans as well as rodents (Schrauwen and Hesselink 2003; Amati et al. 2008). This increased metabolic efficiency and nutrient utilization gained by increased physical activity could have been beneficial for growth efficiency during the reproduction stage, when energy requirements are sustained far above normal requirements. Indeed, highly active females were able to reallocate their energy, which was used normally to sustain physical activity to their offspring by decreasing their physical activity levels. The latter may be facilitated by hormonal changes that occur during lactation. Human studies also supported the notion that moderate exercise training during pregnancy and lactation is beneficial both for mothers and offspring development later in life (Gavard and Artal 2008; Impact of physical activity during pregnancy and postpartum on chronic disease risk2006).

5. Early life influences

Early life experiences are relevant in the context of maintenance of adult health, or disease development later in life. The mechanisms have yet to be resolved, but probably include gene-environment influences. The "thrifty genotype hypothesis" explains that genetic factors only predispose individuals to diseases but environmental factors determine phenotypic expression whether the disease becomes manifest or not (Neel 1999). For example, a thrifty gene enables the organism to store nutrients during times when food is scarce, but in times when it is plenty it leads to excess energy storage and metabolic diseases. In this way, comfort foods could have maladaptive consequences on offspring development and later in life, when availability of food remains and psychological stressors are chronic (van Dijk and Buwalda 2008). According to Chapter 6, palatable high fat/sugar feeding during the perinatal stage causes exaggerated weight gain and adiposity with a larger susceptibility in male offspring than in female offspring (chapter 6). The duration of the diet supply also determines the intensity of derangements (chapter 5), and probably depends on the critical timing of postnatal development of feeding circuits in the central nervous system (Bouret and Simerly 2006). When the maternal HF diet is supplied to the pups via nursing (i.e., feeding via the milk), the offspring show less severe consequences of the maternal HF diet than when they start eating from it by themselves. For example, in the case of a perigestational HF diet condition strictly via the milk, offspring developed increased growth with a proportional increase in fat mass. Nutritional effects on postnatal development in rodents are also dependent on the size of the litter beyond the type of diet. Small litter offspring are more prone to develop metabolic diseases together with elevated plasma insulin and low levels of adiponectin even without disproportional fat mass increases (Chapter 6). On the other hand, the trait for high voluntary activity seems to protect against the deleterious effects of perinatal HF diet feeding with an increased water intake and adiponectin levels. These effects facilitate cell metabolism and energy expenditure (Chapter 6). Although cross-fostering pups between mothers of different lines caused transient changes at adulthood, the trait of high physical activity was not influenced by postnatal environmental conditions, meaning that the protective function of a high activity trait was determined by prenatal (epigenetic) factors (chapter 7). In contrast, control mice fostered by activity selected mothers were sensitive to postnatal influences and developed similar growth, glucose and insulin levels as selected mothers. This is in line with studies showing that maternal physical activity positively influences offspring metabolic- and mental development, and promotes long-term benefit against chronic diseases in the offspring (Impact of physical activity during pregnancy and postpartum on chronic disease risk2006; Gavard and Artal 2008; Larson-Meyer 2002). The task that lies ahead of us is to unravel the mechanisms behind these interactions.

6. Mapping genotype after phenotyping selectively bred animals

Despite our vast knowledge on the beneficial effects of physical activity, and the efforts of healthcare officials, medical specialists, and government programs to promote it, many people still remain physically inactive, and have an increased risk to attract metabolic diseases. Apparently our understanding in the treatment of complex diseases falls short. Studying complex traits such as those of mice selectively bred for increased running wheel activity may provide novel directions for research (see: http://biology.ucr.edu/people/faculty/Garland/Experimental_Evolution_Publications_by_Ted_Garland.html). One of these directions should acknowledge the genetic underpinnings of differences in expression of phenotypes. The level of physical activity has been shown to be determined by many (heritable) genes. More than 214 genes and quantitative trait loci (QTL) have been mapped in humans for performance and health-related phenotypes (Bray et al. 2009), including linkages to physical activity levels (Simonen et al. 2003; Cai et al. 2006). Although the map is exhaustive for currently published accounts of genes and exercise associations and linkages, there are undoubtedly many more gene-exercise interaction effects that have not even been considered thus far, reported by Bray et al (Bray et al. 2009). Recently, the work of Leamy et al provided evidence that epistatic genetic interactions (i.e., when an allele in one locus masks the expression of an allele at another locus) contribute significantly to the variation in physical activity (Leamy, Pomp, and Lightfoot 2008). In other studies, epistatic combinations were found between physical activity and body weight (Leamy, Pomp, and Lightfoot 2009a; Leamy, Pomp, and Lightfoot 2009b), running distance and duration of running (Leamy, Pomp, and Lightfoot 2008; Lightfoot et al. 2008), litter size and maternal performance (Peripato et al. 2002; Peripato et al. 2004). Moreover, some of the QTL discovered for physical activity had pleiotropic effects (i.e., when a mutation of a single gene causes changes in multiple independent phenotypes) on several physical activity related traits in mice selectively bred for high voluntary wheel running (Nehrenberg et al. 2009). Finding these "hot-spots" and how (physiologically/nutritionally or perhaps pharmacologically) and when (i.e., perinatally, during adolescence, or at adulthood) to target them would be of major interest, and could eventually contribute to improvement of health of people in our society.

7. Epilogue.

In modern societies, the competitive and "fast" life-style causes an increase in stressful situations for which physical actions (fight/flight) no longer serve proper solutions. The parallel rise in the availability of comfort foods and automation/industrialization results in multidimensional metabolic diseases. Indeed, in 2005 the World Health Organization estimated the number of obese people worldwide to be 400 million, and they project that in 2015 the number will be as high as 700 million. Obesity is associated with several diseases, for example diabetes mellitus type II, cardiovascular diseases, some types of cancer and musculoskeletal disorders. Cardiovascular

diseases already have the highest death toll and this will continue to rise with the increasing number of people with obesity (World Health Organization 2005, http://www.who.int/). Besides being harmful for health and liveability, the increase in the prevalence of metabolic diseases is also a big economic burden to society. A study by The Conference Board has estimated the costs of obesity in the US to be around $100 billion (The Conference Board, 2008, http://www.conference-board.org/webcasts/describe_wc.cfm?ID=1373). The results described in this thesis may help to understand the protective and preventive nature of physical activity on the growing epidemic of obesity and its related diseases. This not only applies to individuals at adulthood, but particularly to their offspring. Although Westerterp and Speakman argued that human weight gain and obesity over the last decades was not the result of a reduction in energy expenditure, but caused by an increase in energy intake (Westerterp and Speakman 2008; Westerterp and Speakman 2008), healthy daily habits, such as a high degree of physical activity, dietary choice and avoiding anxiety and stress, could perhaps be imprinted during early life conditions. The need for parents to recognize the importance of physical activity and diet during early perinatal development could be supported by widespread means of education.

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NEDERLANDSE SAMENVATTING

"NATURE-NURTURE" EFFECTEN VAN SPONTANE FYSIEKE ACTIVITEIT EN VOEDING OP ENERGIEBALANS REGULATIE EN EMOTIONALITEIT

1. Introductie

Dieren kunnen zich over het algemeen verplaatsen door hun omgeving, hetgeen hen in staat stelt om bepaalde uitdagingen of moelijkheden in hun omgeving uit de weg te gaan (zoals predatie, klimatologische veranderingen, etc.), of juist om bepaalde doelen te verwezenlijken (zoals het vinden van voeding, partners, sociale interacties, etc.). Hierbij verbruiken ze, door spiercontracties en verhoogde activiteit van het cardiovasculair systeem, extra energie boven hun normale energieverbruik. Dit inspanningsgerelateerd verbruik kan per diersoort erg variëren, en zelfs grote variaties kunnen worden gevonden bij verschillende individuen binnen diersoorten. Een toename in de verplaatsingssnelheid of verplaatsbaarheid (locomotie) kan onder bepaalde condities belangrijk zijn, bijvoorbeeld wanneer voedselbeschikbaar in een bepaald gebied plotseling sterk afneemt en dus een groter gebied of "habitaat" moet worden bestreken om te kunnen overleven. Diverse veranderingen in de fysiologie, metabolisme, morfologie, en gedrag van dieren kunnen een positieve bijdragen leveren aan aanpassingen om onder bovengenoemde condities te kunnen overleven als individu en als soort. Het selectief fokken van muizen met verhoogde loopwielactiviteit kan een experimenteel middel zijn om te onderzoeken of dieren met een verhoogde activiteit inderdaad fysiologische, metabole, morfologische en/of gedragsmatige veranderingen laten zien, die mogelijk "adaptief" zijn om een verhoogd activiteitsniveau te kunnen handhaven. In dit proefschrift heb ik gebruik gemaakt van twee muizenlijnen selectief gefokt op hoge loopwielactiviteit, en een controle lijn allen afkomstig uit het laboratorium van Prof dr. T. Garland. In het oorspronkelijke selectiewerk van Garland en collega's werden 4 muizenlijen gefokt op hoge loopwielactiviteit, en 4 controle lijnen waarbij niet werd geselecteerd op loopwielactiviteit. De door ons gebruikte activiteitslijnen (line 7 en lijn 8) vertonen de hoogste loopwielactiviteit, en rennen onder normale condities ongeveer 2.7 keer zoveel in loopwielen dan controle muizen (lijn 2). Ze zijn echter ook een stuk actiever in hun thuiskooi dan controlemuizen wanneer de toegang tot de wielen is geblokkeerd. Het doel van dit promotieonderzoek was om in deze selectielijnen van muizen regulatie van energiebalans en emotionaliteit en evenuele storingen hierin te onderzoeken onder zowel non-reproductieve als reproductieve condities, en om te bestuderen of bepaalde eigenschappen van deze dieren konden worden beinvloed tijdens de perinatale fase. Dit onderzoek is van groot belang voor de gezondheid van de mens aangezien er consensus bestaat over de positieve rol van lichaamsbeweging als middel om overgewicht en daaraan gerelateerde gezondheidsproblemen tegen te gaan. Dergelijke gezondheidsproblemen blijken op steeds jeugdigere leeftijd voor te komen, en zullen mogelijkerwijs een gevolg zijn van processen die tijdens de zwangerschap en de periode vlak na de geboorte "ingeprint" worden door moeder-kind interacties. Dus, door deze processen te volgen in selectielijnen van muizen kon worden onderzocht waar, op welke manier, en wanneer fysieke inspanning of daaraan gerelateerde aanpassingen (fysiologisch, metabool, morfologisch, en/of emotionleel gedragsmatig) bescherming biedt tegen het ontwikkelen van

overgewicht en metabole ziekten. De uitkomsten van de verschillende experimenten zijn hieronder kort samengevat.

2. Samenvatting van de resultaten

In **Hoofdstuk 2** werden emotioneel gedragsmatige consequenties bestudeerd van selectie voor een hoge mate van loopwielactiviteit bij muizen. Daartoe werden muizen zonder loopwielen getest in diverse gestandardiseerde proefopstellingen ("plus-maze, "complex-maze", en "open-field"). Uit de proeven bleek dat hoog actieve muizen een verhoogd niveau van aandacht en angst hadden in een nieuwe omgeving dan controle muizen, maar in een bekende omgeving bleken ze routinematiger te zijn dan controle muizen. Routinematig handelen zou bij kunnen dragen tot een toename in de fysieke activiteit/locomotie onder stress-vrije condities, terwijl een verhoogd aandachtsniveau juist bij kan dragen aan het vroeger en sneller opsporen van bedreigingen en dus verbeterde overleving op onbekend terrein.

In **Hoofdstuk 3** werden hoog actieve en controle muizen voorzien van een gezond vezelrijke vetarm (VA) dieet of een relatief ongezond vezelarm vetrijke (VR) dieet. Na enige weken van habituatie aan deze diëten werd vervolgens onderzocht of de hoog actieve muizen zich op een andere manier aan zouden passen aan het VR dieet dan controles. Uit eerder onderzoek was al gebleken dat de controle muizen op het VR dieet vetzuchtiger werden dan de hoogactieve muizen. In dit experiment bleek dat de laatsten een hogere mate van thuiskooibewegingen lieten zien, en dit bleek verder verhoogd te worden door het VR dieet, terwijl in de controles het bewegingsniveau juist omlaag ging op het VR dieet (t.o.v. het VA dieet). Daarnaast lieten vrouwelijke hoog actieve muizen een toename zien in de vetoxidatie terwijl mannelijke hoog actieve muizen een toename lieten zien van koolhydraatoxidatie. In een dieet voorkeurstest bleek dat m.n. hoog actieve vrouwtjes een sterk verhoogde voorkeur te hebben voor het VA dieet vergeleken met controles. Het is waarschijnlijk dat deze dieren v.w.b. hun koolhydraatstofwisseling op het randje balanceren, en nauwelijks voldoende intermediaire brandstoffen hebben om hun hoog niveau van vetoxidatie op peil te kunnen houden.

In **Hoofdstuk 4** werden hoogactieve muizen en controles voorzien van twee verschillende diëten; wederom een VA dieet, en dit keer een VR dieet tevens bestaande uit een verhoogde concentratie "snelle" suikers (VR/suiker). Na gedurende enkele maanden op de verschillende diëten te zijn geweest bleek dat de hoo gactieve muizen, ondanks een verhoogde opname van het VR/suiker dieet, een verminderde aanleg hadden om vetzuchtig te worden op dit dieet dan controles. Bovendien waren de hoogactieve muizen kleiner dan de controlemuizen. Dit verschil bleek niet te kunnen worden verklaard door een verminderde absorptie, maar door een verhoogde mate van thermogenese gekoppeld aan thuiskooiactiviteit, m.n. wanneer ze op het VR/suiker dieet stonden. Hoogactieve muizen bleken een hogere angst-niveau te hebben in de voor hen onbekende "plus-maze" dan controles, maar dit bleek totaal om te keren wanneer ze het VR/suiker dieet aten. Aangezien de hoogactieve muizen wederom een verhoogde voorkeur

voor het VA dieet hadden dan voor het VR/suiker dieet kon worden geconcludeerd dat het VR/suiker dieet een stemmingsverbeterend effect heeft in de hoogactieve dieren zonder dat de dieren dit dieet daadwerkelijk ook smakelijker vinden.

In **Hoofdstuk 5** werden vrouwelijke hoogactieve en controle muizen wederom voorzien van één van beide diëten als genoemd onder Hoofdstuk 4. Vervolgens werden ze zwanger gemaakt door een mannetje uit overeenkomstige lijn, en werden de reproductieve prestaties onderzocht. Uit de resultaten bleek dat de muizen uit hoog actieve lijnen gemiddeld zwaardere en grotere nesten hadden dan de controle lijn, vooral wanneer ze op het VR/suiker dieet stonden. Tijdens lactatie bleek tevens dat de hoog actieve dieren een verhoogde groei-efficientie (i.e., groei van moeder en pups tezamen tijdens de lactatie gedeeld door de hoeveelheid geabsorbeerde energie) hadden t.o.v. controle muizen, en dit verschil kwam m.n. tot uiting wanneer ze op het VR/suiker dieet stonden. Uiteindelijke werden pups van de controle moeders gemiddeld zwaarder dan die van de hoog actieve moeders, vooral bij diegene op het VR/suiker dieet. Overleving van pups in de hoog actieve lijnen was niet eenduidig, aangezien één van de hoog actieve lijnen (lijn 7) relatief veel pups verloor, terwijl dit juist helemaal niet het geval was in de andere hoog actieve lijn (lijn 8) en controle lijn 2. Regressie analyse liet zien dat effecten op groei van de nakomelingen in grote mate afhankelijk bleek te zijn van nestgrootte waarin de pups werden geboren, maar significante verschillen tussen controle en selectielijnen bleef bestaan.

In **Hoofdstuk 6** werden de lange termijn consequenties onderzocht bij de nakomelingen van de onder Hoofdstuk 5 gemanipuleerde moeders. Ondanks het feit dat alle muizen na het verspenen werden groot gebracht op het VA dieet, bleek het perinatal VR/suiker dieet grotere en dikkere volwassen mannelijke muizen (met hogere insuline, maar niet hogere leptine spiegels) voort te brengen in de controle lijn. Dit effect bleef overeind wanneer werd gecorrigeerd voor verschillen in nestgrootte waarin de dieren werden geboren. Het VR/suiker dieet tijdens zwangerschap en lactatie bleek echter niet tot grotere en zwaardere dieren te leiden in de hoog actieve lijnen. Hieruit kon worden geconcludeerd dat de eigenschap van hoge fysieke activiteit bescherming biedt tegen het perinatale effect van het VR/suiker dieet.

Het doel van **Hoofdstuk 7** was tweeledig: A) Bestudering van de effecten van de twee dieten tijdens het perinatal stadium op regulatie van energiebalans bij de nakomelingen (zoals genoemd onder Hoofdstuk 6), maar nu in de conditie waarin de nestgrootte meteen na de geboorte werd gemanipuleerd naar 10 pups/nest. B) Bestudering van de bijdrage van het postnatale miljeu op energiebalans regulatie door nakomelingen van moeders van verschillende lijnen te verruilen ("cross-fostering"), en dit eveneens in nesten van 10 pups. Uit de experimenten van deel A) bleek dat het perinataal toedienen van het VR/suiker dieet geen noemenswaardige effecten had op energiebalans regulatie van de controle muizen afkomstig uit gemanipuleerde nesten (in tegenstelling tot uitkomsten van Hoofdstuk 6) Nu echter bleek dat de hoog actieve muizen afkomstig van moeders op het VR/suiker dieet zwaarder en dikker waren dan hoogactieve nakomelingen van VA moeders, maar dit effect bleek van tijdelijke aard. Een verklaring van deze uitkomsten kan gevonden worden in het feit dat nesten van controle

moeders, en dan met name diegene op het VR/suiker dieet, vaak een eigen nestgrootte van 7 à 8 pups hadden, en daardoor wellicht geprepareerd waren voor relatief kleine nesten. Aanvullen tot 10/nest leverde daardoor wellicht relatief kleinere/lichtere dieren op. In de hoogactieve muizen was vaak sprake van nesten >10 pups, waardoor moeders na manipulatie dus minder pups hoefden te voeden dan waarvoor ze waren geprepareerd. Dit zou dan kunnen leiden tot relatief grotere pups, en sneller groeiende nakomelingen. Maar, zoals eerder gezegd was dit effect van voorbijgaande aard. Kennelijk zijn hoog actieve muizen in staat om opvetting als gevolg van perinatal manipulatie ("nurture") op te vangen vanwege hun specifieke fysiologische/gedragsmatige eigenschappen ("nature"). In deel B) werd gevonden dat controle pups overgelegd naar hoog actieve lijn 8 moeders geen verschillen lieten zien in groeisnelheid t.o.v. controle pups die bij de eigen moeder bleven. Controle pups overgelegd naar hoog actieve lijn 7 moeders lieten echter een groei achterstand zien t.o.v. de controle pups bij controle moeders. Dit effect bleef zichtbaar in volwassen controle muizen, en was geassocieerd met relatief lage insuline en glucose spiegels. Kennelijk overheerst de relatief "zuinige" postnatale omgeving van hoog actieve lijn 7 moeders de prenatale (epi)genetische "make-up" van controlemuizen. Darentegen kon de (epi)genetische "make-up" van hoog actieve lijn 7 en 8 pups overgelegd naar controle moeders niet worden overheersd door de relatief "rijke" postnatale omgeving van controle moeders. Effecten van "cross-fostering" werden niet gevonden op loopwiel activiteit bij de nakomelingen. Lijn 7 moeders bleken nu geen pups meer te verliezen (dit i.t.t. de experimenten in Hoofdstuk 5), hetgeen mogelijk verklaard kan worden doordat de overgelegde controle pups in deze nesten de zorg van lijn 7 moeders verbeterd. De groei-efficientie bleek te zijn toegenomen in controle moeders en hun nest (m.n. op een VR/suiker dieet), hetgeen mogelijk verklaard kan worden door een hogere groei-efficientie van overgelegde lijn 7 en lijn 8 pups.

3. Conclusie en perspectief

Muizen geselecteerd op een hoge mate van loopwiel activitiet hebben diverse (epi)genetisch vastgelegde fysiologische, morfologische, en gedragsmatige veranderingen (t.o.v. controlemuizen) die hen in staat stellen om actief te zijn in loopwielen, maar waarschijnlijk ook in relatief grote biologische habitaten. Verder zijn de hoog actieve muizen in staat opvetting op een VR/suiker dieet tegen te gaan, zowel in het volwassen stadium als tijdens de perinatale ontwikkeling. Hierbij spelen zowel de (epi)genetsiche als postnatal invloeden een rol. Deze bevindingen kunnen van belang zijn voor de humane situatie. Immers, mensen stammen af van paleolithische voorouders die waarschijnlijk waren geselecteerd en aangepast aan een situatie waarin ze fysiek actief moesten zijn om te kunnen overleven, en daarnaast een relatief karig bestaan hadden. De genetische aanleg van de huidige mens wijkt waarschijnlijk niet erg af van deze voorouders, maar de omgeving darentegen is dramatisch veranderd. In de huidige maatschappij is er sprake van een overvloed aan vaak ongezonde voedingsmiddelen, vermindering van fysieke arbeid (door

toename van transportmiddelen, industrialisatie, en communicatie), en vaak een gejaagde "life-style", waarin stressvolle situaties niet meer beslecht kunnen worden door fysieke energie-kostende acties (zoals waarschijnlijk wel het geval was bij onze paleolithische voorouders), maar vaak chronisch en psychosociaal van aard zijn. Ondanks deze "obesogene" omstandigheden zijn sommige mensen toch in staat om een "normaal" en gezond lichaamsgewicht te handhaven, en geen metabole verstoringen/ziekten te ontwikkelen. Volgens Levine en collega's (Science 28: Vol 307, 5709, pp 584-586, 2005) wordt deze obesitas-resistentie veroorzaakt door verschillen in spontane fysieke activiteit. Westerterp en Speakman (International Journal of Obesity 32: pp 1256-1263, 2008) noemen echter dat toename van voedselinname, en niet energie verbruik, hier een belangrijkere rol in speelt. Toekomstig onderzoek zal moeten uitwijzen welke strategie de beste zal zijn om een verdere toename van metabole welvaartziekten tegen te gaan, en uiteindelijk uit te bannen.

MAGYAR ÖSSZEFOGLALÁS

A TERMÉSZET/GENETIKA ÉS A NEVELÉS HATÁSAI AZ ÖNKÉNTES MOZGÁS AKTIVITÁSRA ÉS TÁPLÁLKOZÁSRA; ENERGIA HÁZTARTÁS ÉS VISELKEDÉS

A mozgás segíti az állatfajt abban, hogy feltérképezzék környezetüket táplálék, folyadék, partner felkutatásának reményében vagy éppen környezeti kihívások elkerülése végett úgy, mint a predáció elkerülése, éghajlati változások, stb. A fizikai aktivitás szintje különbözhet bizonyos állatfajok között, de még egyes állatfajokon belül is. Magas szinten növelheti a túlélés esélyeit azáltal, hogy az állat nagyobb területet képes befedni. Ennek következményeként a tápanyagszükséglet megnövekszik, hogy kielégítse a vázizomzat és a szív-érrendszer energia igényeit. Hosszabb távon ez fiziológiai, morfológiai és viselkedésbeli változásokat okozhat a szervezetben, melyek segítik fenntartani a fizikai aktivitás magas szintjét. Az önkéntes fizikai aktivitással együtt járó lehetséges adaptációs változások tanulmányozására állatkísérletekben kifejlesztett módszer a szelektív tovább-tenyésztés vagy mesterséges szelekció. Ez esetben az állatokat egy bizonyos phenotipikus tulajdonságra szelektálva tenyésztenek, amely szelekció változásokat idéz elő a kiválasztott phenotipusnak alárendelt jellegzetességekre nézve, amik erősítik az egyéni eltéréseket. Prof. T. Garland és kollégái a megnövekedett futókerék hajtásra tenyésztett egereket, amiket négy aktív származási ágra osztott és a randomszerűen tenyésztett 4 másik kontroll ághoz hasonlította őket. E tézis kereteiben a magas mozgásaktivitású egerek két leszármazási ágból kerültek ki (7-es ág és 8-as ág), amiket az egyik randomszerűen tenyésztett kontroll ághoz viszonyítottunk (2-es ág). Célkitűzéseink voltak, hogy vizsgáljuk a magas fizikai aktivitás hatását és adaptációs változásait az energia háztartás szabályozásában és a viselkedésben normál és szaporodási időszakban, valamint, hogy e változások képesek-e kivédeni a zsírdús diéta „obeso-gen" (elhízást okozó) hatásait mind felnőtt- és perinatális táplálás alkalmával. Továbbá, a posztnatális környezet – nevelés fontosságát vizsgáltuk a magas fizikai aktivitás meghatározásában. Az alábbiakban a különböző tanulmányok főbb eredményeit összegeztem fejezetenként.

A **2. fejezet** célkitűzése volt a mozgás aktivitásra vonatkozó szelektációval együtt járó következményes viselkedésbeli változások tanulmányozása. Az eredmények azt mutatták, hogy a magas fizikai aktivitású egerek különbözőképpen viselkedtek új és megszokott környezetben. Új környezetben magasabb anxietással, nagyobb explorációval és kockázat vállalással reagáltak arra utalva, hogy nagyobb óvatossággal és figyelmességgel rendelkeznek a kontroll egerekhez képest. Ezzel ellentétben, megszokott környezetben a rutin jellegű viselkedésük dominált, ami valószínűleg hozzájárul a kifejezettebb állóképességi munkájuk fenntartásához.

A **3. fejezetben** az energia háztartás időbeli változását vizsgáltuk a napi ciklus függvényében szelektált és kontroll egyedeken, amikor magas szénhidrát és alacsony zsírtartalmú (LF) vagy 60%-os, azaz magas zsírtartalmú tápot (HF) fogyasztottak. A kontrollokhoz viszonyítva, a magas fizikai aktivitású egyedek fokozott mozgás aktivitással és

energiafogyasztással/égetéssel rendelkeztek (különösképpen a HF diétán) az aktív fázis alatt. Ez utóbbi a hímekben magas szénhidrát égetésnek felelt meg, míg a nőstényekben ez a fokozott zsírégetés következménye volt, különösképpen a HF diétán. Mivel, a szelektált nőstények a megnövekedett energia szükségletük nagy részét az aktív fázis vége felé elégítették ki, elképzelések szerint ez anyagcsere elégtelenséghez vezethet hosszú távon, ami részben magyarázza soványságukat. Ezek ismeretében a LF diéta preferálása a HF diétával szemben, táplálék választásos kísérletben, meglepő lehet, habár ez anyagcseréjük jelzéseként is felfogható, ami a zsír égetéséhez szükséges szénhidrát szükségességét jelzi. A zsírból származó energia pótlást csak akkor tudják normalizálni, ha elegendő szénhidrátot is fogyasztanak; egy régi mondás szerint is „a zsírok a szénhidrátok tüzében égnek el".

A **4. fejezetben** a LF/normál és finomított cukrot tartalmazó 40%-os HF diéta hatását vizsgáltuk az energia háztartásra és az érzelmi stresszre kontroll és aktív egyedeken. Az eredmények azt mutatták, hogy az aktív egerek rezisztensek voltak az „obeso-gen" HF diétára nézve és magas táplálék felvétellel, hasonló tápanyag felszívódással és magasabb „nem mozgás-indukált termogenezissel" rendelkeztek, mint a kontrollok. Amíg a szelektált nőstényeknek magasabb volt az anxietási szintjük a LF diétán, ez ellentétessé vált a HF diétán, plazma kortikoszteron változás nélkül. Ez talán azt sugallja, hogy a HF diétának hangulat javító szerepe van a szelektív aktív egereken, különösképpen a nőstényeknél, annak ellenére, hogy kevésbé preferálták a HF diétát, mint a kontrollok.

Az **5. fejezetben** a szaporodási képességet vizsgáltuk LF és finomított cukrot tartalmazó 40%-os HF diétán kontroll és aktív egerek között. Az eredmények azt mutatták, hogy az aktív egyedek magas alom-számot prdukáltak, és alom-tömeget szültek, és a növekedésük hatékonysága kiemelkedő volt a kontrollokhoz képest a laktáció időszakának maximumán, amikor HF diétát fogyasztottak. Habár, a szelekciónak negatív következménye volt az utódok korai posztnatális túlélésére a 7-es aktív ágban. Regressziós analízis felfedte, hogy az alom szám egy fontos meghatározó faktor volt az utód jellegzetességekre nézve, a diéta és származási ág hatásaitól függetlenül.

A **6. fejezetben** a terhesség és laktáció alatti HF és LF diéta hosszú távú hatását vizsgáltuk az energia egyensúly szabályozására aktív és kontroll egér utódokban. Az eredmények azt mutatták, hogy az aktív egerek védve voltak a korai HF diéta táplálás káros hatásai ellen. A szelektált egerek megtartották a sovány phenotípusukat emelkedett plazma adiponektin és csökkent plazma inzulin, leptin szintek mellett. Ezzel ellentétben a randomszerűen tenyésztett kontrollok megnövekedett testhosszal, nagyobb zsírtömeggel, emelkedett inzulin és csökkent adiponektin szintekkel rendelkeztek. Regressziós analízis kimutatta, hogy a születési alomszám egy meghatározó faktor felnőttkori paraméterekre nézve is, és a korai diétával és származási ággal együttesen befolyása van a strukturális növekedésre.

A **7. fejezetnek** két fő célkitűzése volt: A) a terhesség és laktáció környéki LF és HF diéta táplálás hatásainak vizsgálata az alomszám különbözőségektől függetlenül a szaporodási teljesítményre és az utód jellegzetességekre nézve; B) posztnatális környezet befolyása az aktív és kontroll egerekben „cross-fostering" típusú kísérletben. E fejezet A) részében az eredmények azt mutatták, hogy az alomszám azonos számra hozatala kivédte a posztnatális perigesztációs HF diéta testtömeg növelő hatását a kontroll utódokban, míg ideiglenesen testsúly és zsírtömeg növekedést okozott az aktív egyedekben, serdülőkorban. A váratlan eredményekre utaló magyarázat az, hogy a csoportok közötti alomszám kiegyenlítődés az utódszám csökkenését okozta az aktív anyáknál, míg a kontrolloknál az utódszám növekedését eredményezte. Így, az aktív anyák átlag két utóddal kevesebbet neveltek fel, ami túltáplálást okozott utódaiban, akiknek testsúlyuk és adipositásuk megnőtt. Ennek ellenére, a kontrollok több utódot neveltek fel, mint amennyire előkészültek a terhesség alatt, és ez az utódok testsúly és zsírtömeg normalizálódását eredményezte. Az aktív utódok túltáplálásának hatása átmeneti volt, amit a magas fizikai aktivitás felülírt felnőttkorban. A fejezet B) részében a cross-fostering kísérlet kimutatta, hogy az aktív anyák különböző posztnatális környezetet biztosítottak utódaiknak, mint a kontroll anyák; habár különbség mutatkozott a két szelektált ág anyái között is. Az utódok kicserélése a kontroll és a 8-as ág aktív anyái között nem volt befolyással az utódok növekedésére. Viszont az utódok kicserélése a kontroll és a 7-es ág aktív anyái között, a kontroll utódok testsúly csökkenését, és a 7-es aktív utódok időleges testsúly növekedését okozta. Az aktív anyák posztnatális környezete alacsony plazma inzulin és glukóz szinteket eredményezett a felnőtt kontroll utódokban, függetlenül a diéta hatásától, hatástalanítva a kontroll utódok prenatális (epi)genetikai hátterét. Ettől ellentétesen a felnőtt szelektált utódok prenatális genetikai hátterét nem tudta felülmúlni a kontrollok aránylag gazdagabb posztnatális környezete, még a perigesztációs HF diéta táplálása esetén sem. Érdekes módon, a kontroll anyák és utódaik növekedési hatékonysága kiemelkedő volt a laktációs időszakban, amikor HF diétát fogyasztottak. Ez az eredmény különbözött az 5. fejezetben találtaktól, ami arra enged következtetni, hogy a fogadott aktív utódok feljavították a növekedés hatékonyságát a kontroll ágban. Továbbá az 5. fejezetben talált 7-es ág aktív anyáinak utód vesztesége nem ismétlődött meg e fejezet 7-es ág anyáinál, akik kontroll utódokat is neveltek. A feljavult utód túlélés valószínűleg azt mutatja, hogy az utódveszteség az anya-utód kétirányú kapcsolat következménye és nem a gyengült anyai gondoskodás által volt megfigyelhető.

A mai korszerű társadalmakban a versenyszerű és felgyorsult életstílus feszültségekkel teli szituációkat okoz, amihez a következményes stressz reakció (fight or flight) nem szolgál megfelelő megoldással. Ezzel párhuzamban a táplálék megnövekedett mennyisége és megromlott minősége, a gépesítés/indusztrializáció mind multi-dimenzionális megbetegedéseket eredményez. 2005-ben a Világ Egészségügyi Szervezetének (WHO) felmérése 400 millióra becsülte az elhízottak számát világszerte, ami 2015-re kivetítve elérheti a 700 milliót. Sok esetben, az elhízás együtt jár különböző megbetegedésekkel úgy, mint a 2-es típusú cukorbetegség, cardiovasculáris

megbetegedések, rákos daganatok bizonyos típusai és váz-izomrendszeri elváltozások. A cardiovasculáris megbetegedéseknek van a legmagasabb halálozási aránya, ami tovább nő az elhízás fokozott előfordulásával (World Health Organization 2005, http://www.who.int/). Mindezeken túl a kapcsolt metabolikus betegségek magas pénzügyi terhet jelentenek. A Conference Board egy tanulmánya az elhízás költségeit 100 milliárd dollárra becsülte az Egyesült Államokban (The Conference Board, 2008, http://www.conference-board.org/webcasts/describe_wc.cfm?ID=1373).

E tézisben leírt eredmények remélhetően segítséget nyújtanak a mozgás aktivitás természetes védő és megelőző szerepének megerősítésére az egyre növekvő elhízás és társbetegségeinek megelőzésében és kezelésében. A mozgás aktivitás jótékony hatása nemcsak felnőtt egyénekre vonatkozik, hanem egyre fokozottan a gyermekekre is. Westerterp és Speakman megállapították, hogy a múlt évtized testsúly növekedése és a fokozott elhízás nem az energia felhasználás/égetés csökkenésének tulajdonítható, hanem az energia felvétel/táplálkozás növekedésének. Ezenfelül kimutatott, hogy a korai és gyermekkori életkörülmények befolyásolhatják a felnőttkori egészséges napi ritmus kialakítását úgy, mint a rendszeres mozgásaktivitást, megfelelő táplálék választást és a stresszes életritmus elkerülését. Ezért, a szülők felvilágosítása a megfelelő mozgás aktivitás és helyes táplálkozás fontosságára nézve támogatásra szorul széleskörű oktatással.

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