Diet-induced obesity resistance of adult female mice selectively bred for increased wheel-running behavior is reversed by single perinatal exposure to a high-energy diet

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1. Introduction

Obesity is a risk factor for impairment of sustainable health, as it increases susceptibility for developing type-2 diabetes, cardiovascular diseases, cancer, and neurodegenerative diseases [1, 2]. Although the energetics underlying obesity are rather straightforward - it results from a mismatch between intake and expenditure - the underlying mechanisms probably include a multitude of interactions between environmental and genetic factors [3]. Sensitivity to obesity in humans has long ago been reported to have a high degree of inter-individual variation [4, 5], yet the underlying mechanisms are still unclear [4]. The increasing incidence of childhood obesity [6] points towards early stages of life as a critical window for acquisition of predisposition to gain weight later in life [7–9]. Indeed, several animal studies have shown that over-nutrition by a high-energy (HE) diet during pregnancy and lactation predisposes offspring to energy-balance disorders and cardio-metabolic derangements later in life [10–15]. Early nutritional influences on long-term health outcomes have been named “fetal programming” [7, 16] or developmental plasticity [17]. From an evolutionary point of view, tendency for weight gain is regarded as an adaptive strategy to secure nutrients in order to survive periods of famine [18].

Besides energy intake, another variable component affecting energy balance is metabolic rate (MR). Metabolic rate increases with physical

Abbreviations: CON, not selected control line; DIO, diet induced obesity; HE, high energy; LF, low fat; MEO, milk energy output; MR, metabolic rate; OGTT, oral glucose tolerance test; PA, physical activity; RMR, resting metabolic rate; RQ, respiratory quotient; S, mice selectively bred for increased voluntary wheel-running behavior.
activity (PA), which can account for 20–40% of total MR in humans [19]. PA greatly varies among individuals within species as well as between species [20]. One possible factor underlying this variation is that certain personality traits are associated with different levels of voluntary PA in humans [21, 22], as well as in other animals [20, 23, 24]. One approach to study the neurobiology of “innate” PA and its role in energy balance and adiposity is to investigate these regulations in mice from lines selectively bred for high voluntary wheel-running behavior [20, 25]. In previous studies, it was found that relative to mice from non-selected control lines, activity-selected mice have reduced body mass, reduced body fat content, increased mass-adjusted food consumption, increased daily MR, and a high maximal oxygen consumption during forced treadmill exercise [26–28]. These traits are expressed even if mice do not have access to running wheels [29, 30]. When exposed to a high energy (HE) diet in adulthood, the hyperactivity trait appears to protect female mice from diet-induced obesity (DIO), despite the fact that they are markedly hyperphagic compared to CON mice that do become obese [26]. Resistance in highly active females is, in part, attributable to diet-induced augmentation of voluntary PA, as well as several metabolic and endocrine changes that stimulate fuel metabolism [26]. Whether these mice are also protected against DIO and development of metabolic derangement when subjected to developmental programming effects by a HE diet is unknown. For this reason, male and female mice from a non-selected control line (CON) and from two of the selectively bred high-activity lines (S1 and S2) were subjected to combinations of perinatal and/or post-weaning HE diet exposure (45% fat and 13% added sucrose) or a low-fat diet (and lower in energy content) consisting of 13% fat and no added sugars. The consequences of these combinations for weight gain, adiposity levels, fuel homeostasis, voluntary PA, and wheel-running behavior were investigated. Based on the robustness of the high-activity trait in several other studies (see [18] for comprehensive review), we hypothesized that the S1 and S2 mice, unlike the CON mice, would resist combinations of perinatal and post-weaning HE diet-induced changes in the aspects of energy balance mentioned above.

2. Material and methods

2.1. Animals and housing

Mice from one Control (CON, lab-designated line 2) and from two lines (here S1 and S2, lab-designated, respectively, line 7 and 8) obtained from the same ancestral line but selectively bred for high wheel-running activity were used (starting population for all lines was outbred Hsd:ICR mice). They were 53rd-generation offspring that were obtained from T. Garland Jr., Riverside, CA, USA [31]. At generation 45, ten pairs from CON, S1, and S2 lines were shipped to Groningen, the Netherlands, and maintained at the University of Groningen animal facility and further selected for high wheel-running activity. We studied two selected lines since by artificial selection phenotypic changes in one trait can impact on the expression of other traits. Hence, mice in different lines may have different adaptations to sustain the behavior they are selected for.

Three cohorts of CON, S1 and S2 mice were bred each using 20 virgin females per line, of which half received a low-fat diet (LF; 15.9 kJ/g, 13% fat, 63% starch 24% protein, RMH-B 2181, HopeFarms BV, Woerden, NL) and the other half a high-energy diet (HE; 19.7 kJ/g, 45% fat, 18% starch, 13% sucrose, 24% protein). After being on diets for two weeks, each female was paired for two weeks with a male from the same line (and thus ate from the same diet as the female). After delivery, litters were not culled and kept unaltered after birth, because there is no way of knowing whether culling itself would interact with the effect of line and/or diet on several parameters. We nevertheless only used offspring in litters of six or larger, and that consisted of at least two males and at least two females.

All mice were generally housed in Plexiglas cages (Macrolon Type II, UNO Roestvaststaal BV, Zevenaar, NL), with food and water ad libitum, and nesting material (EnviroDry®) as bedding. They were kept on a 12:12 light-dark cycle (lights on: 9 am) at 22 ± 1 °C. All experiments were approved by the Animal Experimental Committee of the University of Groningen.

2.2. Perinatal effects of diet

In breeding cohort 1, offspring were sacrificed at PND21, and the perinatal (i.e., here defined as the period from ~2 weeks before pregnancy until PND 21) effects of HE diet vs LF diet on several parameters were assessed. Half the mice were used for carcass analysis, in which liver, retroperitoneal and gonadal fat pads, gastrointestinal tract, kidneys, and skin including subcutaneous fat were removed and weighed. Carcasses and organs were weighed and then dried for 4 h at 103 °C. Fat was removed from carcasses and organs using petroleum ether extraction. In the other half of cohort 1, gonadal adipose tissue was submerged in 4% paraformaldehyde solution for fixation and embedded in paraffin for histology. In total, 103 white adipose tissue samples were sliced (thickness 4 μm) using a microtome and placed on adhesive glasses each containing 2–4 slices. After deparaffinization by xylol, alcohol, and deionized water, slices were stained for hematoxilin and covered using Kaiser’s glycerin. Pictures were taken using microscope software Leica Qwin V3, creating an image in which areas of one pixel corresponds with 0.1971 μm². Three pictures were taken from representative adipose tissue areas, and analyzed using ImageJ (http://rsbweb.nih.gov). From each section, exactly 620 cells were randomly chosen and sizes were determined. We excluded objects >240 μm and we measured shapes with a circularity between 0.3 and 1. Due to the skewness of distribution, all adipocytes of all mice irrespective of group were pooled and ranked according to size from small to large and then reassigned to groups. Rank numbers where averaged for each group.

2.3. Post-weaning effects of diet

In cohorts 2 and 3, two males and two females from each litter were weaned at PND21 and singly housed. To control for maternal effects, one male and female from each litter were subjected to LF diet whereas the other male and female of the same litter were subjected to HE diet. Remaining mice were used to continue the breeding lines. In each gender and line, this resulted in four dietary groups: LF-LF, HE-HE, HE-LF and HE-HE (first abbreviation: perinatal diet; second abbreviation: post-weaning diet). Cohort 2 had no access to running wheels and underwent several tests as described below. Only cohort 3 was studied for wheel-running activity between 6 and 8 weeks and at 10 months of age according to methodologies described elsewhere [31].

2.4. Food intake, bomb calorimetry, and home-cage activity

Food intake over seven days in cohort-2 offspring was recorded at four and eight months of age. After receiving clean cages, amount of food consumed (corrected for spillage) was determined (to the nearest 0.05 g). Furthermore, feces were collected and its energy content was measured by a bomb calorimeter (CBB 330, standard benzoic acid 26.44 kJ/g). Absorbed energy was calculated from differences between energy intake and fecal energy content. At four months of age, home-cage activity was measured by passive infrared sensors (Optex Wonderex FX-35) over four days [26].

2.5. Indirect calorimetry

Directly after food intake and home-cage activity measurements, cohort 2 offspring were placed in indirect calorimeter chambers for 24 h (with food and water available). Home-cage bedding was added to minimize stress of novelty. Gas exchange measurements were performed according to methodologies explained in detail elsewhere [32]. As described above, physical activity was measured by passive infrared sensors. Respiratory quotient (RQ) and metabolic rate (MR, kJ h⁻¹) were
Table 1
Body mass, energy intake and absorption efficiency in male control (CON) and running wheel selected (S1 and S2) offspring subjected to combinations of low fat (LF) and high energy (HE) diet conditions during the perinatal and post-weaning stage.

<table>
<thead>
<tr>
<th>Diet</th>
<th>LF-LF</th>
<th>LF-HE</th>
<th>HE-LF</th>
<th>HE-HE</th>
</tr>
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<tbody>
<tr>
<td>Body mass (g)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>21 days</td>
<td>12.6 ± 1.0</td>
<td>16.4 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.6 ± 1.3</td>
<td>13.3 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 month</td>
<td>22.5 ± 1.7</td>
<td>21.3 ± 1.4</td>
<td>27 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 months</td>
<td>38.8 ± 1.6</td>
<td>37.8 ± 1.1</td>
<td>41.8 ± 1.1</td>
<td>43.7 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>7 months</td>
<td>41.1 ± 1.8</td>
<td>42.2 ± 2.3</td>
<td>43.2 ± 1.6</td>
<td>49.5 ± 3.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 months</td>
<td>41.1 ± 1.9</td>
<td>41.9 ± 2.4</td>
<td>44.3 ± 1.8</td>
<td>50.3 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Energy intake (kJ)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 months</td>
<td>69.4 ± 6.9</td>
<td>85.7 ± 13.3</td>
<td>74 ± 3.6</td>
<td>44.2 ± 4.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>8 months</td>
<td>70 ± 1.9</td>
<td>72 ± 1.2</td>
<td>70.8 ± 1.3</td>
<td>78.2 ± 1.3</td>
</tr>
<tr>
<td>Feces Amount (g)</td>
<td></td>
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<tr>
<td>4 months</td>
<td>3.65 ± 0.18</td>
<td>2.58 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.83 ± 0.16</td>
<td>2.55 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Energy (kJ/g)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>4 months</td>
<td>16.71 ± 0.05</td>
<td>17.42 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.74 ± 0.06</td>
<td>17.19 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Energy content (kJ)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>4 months</td>
<td>60.9 ± 2.9</td>
<td>45 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64 ± 2.7</td>
<td>44 ± 4.4&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Efficiency (%)</td>
<td></td>
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<tr>
<td>4 months</td>
<td>83.8 ± 1</td>
<td>86 ± 0.5</td>
<td>83.6 ± 0.3</td>
<td>87.2 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. p < 0.05 (for ANOVA, see Results section).
<sup>a</sup> Perinatal HE contrasts.
<sup>b</sup> Post-weaning HE diet contrasts in LF-LF vs LF-HE and HE-LF vs HE-HE conditions (the first abbreviation refers to the perinatal stage and the second to the post-weaning stage).
calculated accordingly to [33], and resting metabolic rate (RMR) was estimated by averaging the lowest three 10-minute running means during resting for at least 30 min. MR exceeding RMR was assigned physical activity metabolic rate (PA-MR).

2.6. Oral glucose tolerance test

Glucose homeostasis was assessed by an oral glucose tolerance test (OGTT) in cohort 2 offspring at four and eight months. Before testing, mice were food-deprived for 6 h. Blood samples were collected just prior, and at 30, 60, and 120 min after oral glucose administration (2 g/kg, -0.2–0.6 ml, per os, t = 0 min). Blood samples were taken via a small incision of the lateral tail vein. Blood glucose levels were determined using glucose test strips (OneTouch®, LifeScan Inc., Johnson & Johnson) and a glucose meter (OneTouch® UltraEasy™). Plasma insulin levels were determined in blood samples (50 μl) taken at baseline, and 30 and 60 min after glucose administration. After centrifugation (2600 G, 5 min at 4 °C), plasma was transferred into small vials and stored at −80 °C until insulin analysis (RIA kit #RL-13K Linco Research, Nucli lab, The Netherlands). After the last sample, food was

Fig. 1. Body adiposity and plasma leptin at PND 21. Effects of perinatal HE diet in male (left) and female (right) offspring in control (CON) and selected (S1, S2) lines on body adiposity (A), circulating plasma leptin levels (B), gonadal fat (C), and adipocytes size rank (D). The diet conditions “low fat” (LF) and “high energy” (HE) are indicated by open bars and closed bars, respectively. Data are mean ± SEM. *HE diet contrasts (p < 0.05) (for ANOVA, see Results section).
returned to the mice. Area under the curve (AUC) for glucose and insulin were calculated using the trapezoid method.

2.7. Body composition and endocrine/metabolic analysis

At ten months of age, cohort 2 offspring were anaesthetized using isoflurane inhalation. A – 0.5 ml blood sample was taken by heart puncture. The syringes were primed with 10 μl EDTA (9 g/100 ml) to avoid clotting. Samples were centrifuged (2600g, 15 min at 4 °C), plasma was collected and stored at −80 °C until analysis of plasma leptin and insulin (Linco Research, Nucli lab, The Netherlands #RL-83 K for leptin, #RL-13 K for insulin). Liver, retroperitoneal and gonadal fat pads, gastrointestinal tract, kidneys, and skin including subcutaneous fat were removed from carcasses and weighed. Directly after harvesting, a chunk of liver was rapidly frozen in liquid nitrogen, crushed, and homogenized in ice-cold PBS. Hepatic lipids were extracted according to Bligh and Dyer [34] and hepatic triglycerides were measured using a kit from Roche (Mannheim, Germany). Carcass compositions were analyzed as described earlier [26].

2.8. Statistical analysis

Data were analyzed by ANOVA to investigate main effects and interactions of these. When appropriate, body mass was added as covariate in the analysis. Repeated-measures ANOVA was used when time interactions of these. When appropriate, body mass was added as covariate. In addition, Student t-tests were used to explore differences between the perinatal HE, *post-weaning HE conditions. When the dams were exposed to the perinatal HE diet (male: F(1,79) = 25.87, p < 0.001; female: F(1,74) = 29.7, p < 0.001) (tables 1 and 2). Closer analysis of cohort 1 offspring at PND21 revealed that percentage body adiposity (male: F(1,44) = 27.40 p < 0.001; female: F(1,43) = 50.92, p < 0.001) and gonadal fat weight (male: F(1,44) = 9.83, p = 0.003; female: F(1,43) = 17.50, p < 0.001) were all greatly augmented by perinatal HE diet irrespective of sex and line (Fig. 1, panels A and C). Plasma leptin levels, however, were only increased by perinatal HE diet in S1 and S2 male (interaction: F(2,44) = 3.75, p = 0.031) and female (interaction: F(2,43) = 3.32, p = 0.046) offspring, an effect not observed in CON offspring (Fig. 1, panel B). In male offspring of all three lines, gonadal adipocyte sizes were increased by perinatal HE diet (F(1,47) = 48.30, p < 0.001), but in female offspring this effect was only observed in the S1 and S2 lines (interaction: F(2,44) = 3.61, p = 0.035) (Fig. 1, panel D).

3. Results

3.1. Perinatal period

Effects of breeding CON, S1 and S2 lines for the present study resulted in gestation and offspring yields largely comparable to those observed in our previous study on lactation energetics with similar line and diet conditions [37]. Comparable to our previous findings, average number of pups and male/female ratio per litter of dams in the separate lines or diet groups did not differ, which implies that this could not explain potential differences in offspring development among lines and diet groups. Nevertheless, only in the case litters consisted of at least 2 males and 2 females, and at least counting 6 pups per litter, we assigned 1 male offspring and 1 female offspring to the LF diet condition and the other male and female to the HE diet condition after weaning. In every condition, group sizes ranged between 5 and 8 mice.

Over the course of lactation, we previously found that the HE diet exposure greatly increased milk energy output (MEO) and litter growth across all lines compared to the LF diet [37]. This led to increased body mass of mice in all lines and both sexes at PND21 in the present study when the dams were exposed to the perinatal HE diet (male: F(1,79) = 25.87, p < 0.001; female: F(1,74) = 29.7, p < 0.001) (tables 1 and 2). Closer analysis of cohort 1 offspring at PND21 revealed that percentage body adiposity (male: F(1,44) = 27.40 p < 0.001; female: F(1,43) = 50.92, p < 0.001) and gonadal fat weight (male: F(1,44) = 9.83, p = 0.003; female: F(1,43) = 17.50, p < 0.001) were all greatly augmented by perinatal HE diet irrespective of sex and line (Fig. 1, panels A and C). Plasma leptin levels, however, were only increased by perinatal HE diet in S1 and S2 male (interaction: F(2,44) = 3.75, p = 0.031) and female (interaction: F(2,43) = 3.32, p = 0.046) offspring, an effect not observed in CON offspring (Fig. 1, panel B). In male offspring of all three lines, gonadal adipocyte sizes were increased by perinatal HE diet (F(1,47) = 48.30, p < 0.001), but in female offspring this effect was only observed in the S1 and S2 lines (interaction: F(2,44) = 3.61, p = 0.035) (Fig. 1, panel D).
groups $p < 0.05$ by planned comparisons). When body mass was used as a covariate, only post-weaning HE diet increased body adiposity in males of all lines (CON: $F_{(1,24)} = 11.93$, $p < 0.001$; S1: $F_{(1,25)} = 13.66$, $p < 0.001$; S2: $F_{(1,26)} = 10.8$, $p < 0.005$), but again highest levels were found in the HE-HE group (see Fig. 2, left panel). The generally increased body fat percentage was due to increased fat amounts in various compartments that contributed to different extents among the different lines (shown in detail in Table 3). In female CON offspring, post-weaning HE diet increased body fat content ($F_{(1,26)} = 13.49$, $p < 0.001$) irrespective of the perinatal diet, which persisted when body mass was added as a covariate ($F_{(1,25)} = 6.79$, $p < 0.05$). An increase in body fat content by post-weaning HE diet was found in S lines as well, but only in combination with perinatal HE diet ($p < 0.05$, compared to all other groups in S1 and S2). Again, these effects persisted when body mass was used as a covariate ($S1$: $F_{(1,20)} = 7.47$, $p < 0.05$; $S2$: $F_{(1,27)} = 6.13$, $p = 0.019$) (see also Fig. 2, right panel). With respect to individual fat depots, different perinatal and post-weaning HE diet effects were found depending on line and sex; however, highest levels were generally observed in HE-HE offspring (see Table 4).

Plasma leptin levels at sacrifice generally tracked differences in body fat content such that all male offspring irrespective of line experienced at least strong trends for increased plasma leptin levels by post-weaning HE diet (Table 3). In CON and S1 male offspring, plasma leptin levels were further increased by perinatal HE diet (CON: $F_{(1,25)} = 7.21$, $p < 0.05$; S1: $F_{(1,26)} = 6.75$, $p < 0.05$). In female offspring (Table 4), plasma leptin levels increased by post-weaning HE diet. In S2 females, this increase was only observed when post-weaning HE diet was combined with perinatal HE diet ($F_{(1,27)} = 4.45$, $p < 0.05$). In S1 females a trend for increased leptin levels was found in the post-weaning HE diet condition ($F_{(1,25)} = 3.30$, $p = 0.08$).

In CON ($F_{(1,25)} = 4.48$, $p < 0.05$) and S1 ($F_{(1,26)} = 12.55$, $p < 0.01$) male offspring, perinatal HE diet increased liver mass, an effect that was not observed in S2 males. Males subjected to combined perinatal and post-weaning HE diet had increased total hepatic triglycerides content (interaction effect: $F_{(1,25)} = 4.85$, $p = 0.037$). In S1 ($F_{(1,25)} = 13.38$, $p < 0.01$) and S2 ($F_{(1,25)} = 12.81$, $p < 0.01$) male offspring, post-weaning HE diet increased hepatic triglyceride levels. In female offspring, S1 ($F_{(1,21)} = 10.35$, $p < 0.01$) and S2 ($F_{(1,27)} = 5.18$, $p < 0.05$) mice had lower liver masses by post-weaning HE diet, while there were no significant effects in CON female offspring. However, post-weaning HE diet caused increased hepatic triglyceride content in CON and S2 female offspring (resp. $F_{(1,26)} = 14.29$, $p < 0.001$; $F_{(1,27)} = 5.75$, $p < 0.05$), with a trend in S1 female offspring ($F_{(1,21)} = 3.11$, $p = 0.09$) in the same direction.

### 3.2.2. Energy intake, metabolism and home-cage activity

At four and eight months of age, offspring in all diet groups and lines displayed comparable daily energy intake. Overall, post-weaning HE diet caused mice to produce less feces ($F_{(1154)} = 188.57$, $p < 0.05$) with greater energy content than the post-weaning LF diet, and with an overall higher absorption efficiency ($F_{(1154)} = 53.10$, $p < 0.05$, Tables 1 and 2). No main effects were found of perinatal HE diet on energy content of the feces or absorption efficiency at adulthood.

Indirect calorimetry (IC) for assessment of metabolic rate (MR) was performed at four months of age. Because we also assessed behavioral activity (by passive infrared motion detection), dissociation between MR and resting MR (RMR) could be detected. In male offspring, subtle perinatal and post-weaning diet interactions on MR and RMR were performed at four months of age. Because we also assessed behavioral activity (by passive infrared motion detection), dissociation between MR and resting MR (RMR) could be detected. In male offspring, subtle perinatal and post-weaning diet interactions on RMR and MR were detected at adulthood. In female offspring (Table 4), RMR above RMR (which is energy largely devoted to physical activity, interaction effect: $F_{(1,25)} = 4.83$, $p < 0.05$) in female S1 offspring. In female CON and S2 offspring, on the other hand, a post-weaning HE diet effect was observed to increase RMR.

### Table 3

| Body composition and plasma leptin analysis for male control (CON) and running wheel-selected (S1 and S2) offspring at 11 months of age, subjected to combinations of low fat (LF) and high energy (HE) diet conditions during the perinatal and post-weaning stage. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | LF              | HE              | LF              | HE              | LF              | HE              |
| Body mass (g)   | 41.1 ± 1.9      | 41.9 ± 2.4      | 44.3 ± 1.8      | 50.3 ± 2.2      | 36.9 ± 1.5      | 40.1 ± 2.6      |
| Retroperitoneal fat (g) | 0.4 ± 0.1 | 0.5 ± 0.1 | b 0.6 ± 0.1 | 1.1 ± 0.1 | 0.3 ± 0 | 0.7 ± 0.2 |
| Liver fat (g)   | 0.1 ± 0.1       | 0.1 ± 0.1       | 0.1 ± 0.1       | 0.2 ± 0.1       | 0.1 ± 0.1       | 0.1 ± 0.1       |
| Intestinal fat (g) | 0.4 ± 0.1 | 0.5 ± 0.1 | 0.6 ± 0.1 | 0.7 ± 0.1 | a,b 0.2 ± 0.1 | 0.5 ± 0.1 |
| Subcutaneous fat (g) | 1.6 ± 0.3 | 2.1 ± 0.5 | b 1.8 ± 0.3 | 3.4 ± 0.3 | b 1.2 ± 0.2 | 2.5 ± 0.6 |
| Liver (g)       | 1.80 ± 0.11     | 1.57 ± 0.12     | 1.87 ± 0.07     | 1.90 ± 0.06     | 1.47 ± 0.07     | 1.50 ± 0.09     |
| Leptin (ng/ml)  | 2.9 ± 0.3       | 4.7 ± 0.6       | 3.5 ± 0.7       | 7.8 ± 1.2       | 2.5 ± 0.2       | 5.4 ± 0.8 |

Data are mean ± SEM. $p < 0.05$ for ANOVA (Results section).

- Perinatal HE contrasts.
- Post-weaning diet contrasts in LF-LF vs LF-HE and HE-LF vs HE-HE conditions (the first abbreviation refers to the perinatal stage and the second to the post-weaning stage).
### Table 5

Metabolic rate in assessed by Indirect Calorimetry in control (CON) and running wheel selected (S1 and S2) offspring at 4 months of age subjected to combinations of low fat (LF) and high energy (HE) diet conditions during the perinatal and post-weaning stage.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>S1</th>
<th>S2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LF-LF</td>
<td>LF-HE</td>
<td>HE-LF</td>
</tr>
<tr>
<td><strong>Body mass (g)</strong></td>
<td>31.8 ± 1</td>
<td>37.6 ± 2.5</td>
<td>36.5 ± 1.2</td>
</tr>
<tr>
<td><strong>Lean dry mass (g)</strong></td>
<td>8.2 ± 0.2</td>
<td>8 ± 0.3</td>
<td>8.7 ± 0.2</td>
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<tr>
<td><strong>Body fat (g)</strong></td>
<td>3.5 ± 0.4</td>
<td>7.9 ± 1.7b</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td><strong>Gonadal fat (g)</strong></td>
<td>0.6 ± 0.1</td>
<td>2 ± 0.6</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td><strong>Subcutaneous (g)</strong></td>
<td>0.2 ± 0.1</td>
<td>1 ± 0.3b</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td><strong>Liver (g)</strong></td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td><strong>Intestinal fat (g)</strong></td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td><strong>Liver (g)</strong></td>
<td>1.5 ± 0.08</td>
<td>1.36 ± 0.11</td>
<td>1.48 ± 0.09</td>
</tr>
<tr>
<td><strong>Triglycerides (mM)</strong></td>
<td>21.4 ± 2.4</td>
<td>37.9 ± 8.3b</td>
<td>20 ± 4.1</td>
</tr>
<tr>
<td><strong>Leptin (ng/ml)</strong></td>
<td>3.1 ± 0.6</td>
<td>4.9 ± 1.6</td>
<td>3.1 ± 0.5</td>
</tr>
</tbody>
</table>

Body mass (BM), metabolic rate (MR), resting metabolic rate (RMR), metabolic rate above resting metabolic rate (PA-MR), and respiratory quotient (RQ) are expressed as mean ± SEM. *p < 0.05 (for ANOVA, see Results section).

* Perinatal HE contrasts.

** Post-weaning HE diet contrasts in LF-LF vs LF-HE and HE-LF vs HE-HE conditions (the first abbreviation refers to the perinatal stage and the second to the post-weaning stage).
When S1 and S2 lines were compared we found a significant line* perinatal *post-weaning interaction (F(1,54) = 5.80, p < 0.05) in female offspring, showing that S1 females increased MR when given a HE diet. This compensatory increase of MR in S1 females was lost when their mothers were subjected to the HE diet. Contrary to S1 females, S2 females presented a further increased MR when experiencing the combined perinatal and post-weaning HE diets.

Planned comparison of home cage PA over an 8-day period shortly before IC comparing LF-LF offspring between lines revealed that PA activity of S1 (157%) and S2 (82%) females was higher than in CON females. In LF-LF male offspring, S2 males had 66% higher home-cage activity levels compared to CON males, but no difference was observed in S1 males relative to CON males. Perinatal HE diet caused decreased home cage activity in male CON offspring (F(1,28) = 9.88, p < 0.01) (see Fig. 3). In contrast, post-weaning HE diet caused decreased home cage activity only in female CON offspring (F(1,28) = 19.66, p < 0.001). A trend for decreased home cage activity was also observed in S1 females.

### 3.2.3. Glucose homeostasis

Oral glucose tolerance tests (OGTT) were performed at 4 and 8 months of age, and glucose and insulin responses in plasma were analyzed and depicted as area under the curve (AUC) in Fig. 4. Because results at 4 months were in the direction of those found at 8 months, only those of the latter time point are shown. In males, perinatal or post-weaning HE diet effects on AUC-glucose were only observed in S2 mice, in which post-weaning HE diet increased AUC-glucose (F(1,31) = 12.77, p = 0.001). With respect to the glucose-induced insulin responses in males, generally the highest levels were observed in the HE-HE groups (by planned comparison). In CON males, AUC-insulin was affected by perinatal (F(1,28) = 6.85, p = 0.015) and post-weaning (F(1,24) = 13.08, p = 0.001) diet. Furthermore, perinatal HE diet affected AUC-insulin in S2 offspring (F(1,30) = 7.23, p = 0.012), and post-weaning HE diet affected AUC-insulin in S1 offspring (F(1,25) = 6.42, p = 0.018). In females, no overall main statistical significant diet effects on AUC-glucose were observed. Overall S2 females had higher glucose response than S1 females (line F(1,48) = 4.88, p < 0.05). With respect to the glucose-induced insulin response, only the HE-HE group in the S1 females showed a clear increase in the AUC-insulin relative to groups. Perinatal (F(1,24) = 4.47, p = 0.045) and post-weaning HE diet (F(1,24) = 19.48, p < 0.001) both increased AUC-insulin in CON mice, without an interaction. Additional post-weaning HE diet effects to increase AUC-insulin were observed in S1 (F(1,21) = 6.13, p = 0.022), but not S2 females.

### 3.2.4. Wheel-running activity

Specific effects of dietary interventions on running wheel activity were analyzed in cohort 3 mice at 6–8 weeks and at 10 months of age. These mice were not used for measurement of energy balance parameters. In cohort 3 LF-LF male offspring at 6–8 weeks of age, wheel-running activity was solely decreased in S2 offspring (+146%) compared to CON offspring. In LF-LF females, wheel-running was higher both in S1 (+92%) and S2 (+100%) females compared to LF-LF CON females. With regards to the comparison between selected lines S1 males had lower running wheel activity than in S2 mice (line F(1,54) = 12.41, p < 0.001). Only female CON offspring showed a marked increase in wheel-running when feeding post-weaning HE diet (6–8 weeks F(1,28) = 19.66, p < 0.001) and this effect was only found at 6–8 weeks of age. All mice had reduced wheel-running behavior when re-tested.
at 10 months, independent of dietary condition. This effect was most pronounced in female CON offspring, where a stronger reduction was observed by post-weaning HE diet (time * diet: F(1,28) = 19.27, p < 0.001) (see Fig. 5).

4. Discussion

Female mice selectively bred for increased voluntary wheel-running behavior (S) were previously shown to resist (unlike randomly selected controls; CON) diet-induced obesity (DIO) and associated metabolic derangements when challenged with a HE diet during adulthood, even without access to running wheels [26]. A combination of neuroendocrine, metabolic, and behavioral mechanisms that co-evolved over the course of ~50 generations of selective breeding for high wheel-running behavior probably underlies this resistance. Here we report that DIO resistance to HE diet exposure from weaning onwards (postnatal day 21) in adult female S mice is lost when they were born from S dams that were additionally exposed to a HE diet from 2 weeks before they were pregnant until end of lactation (i.e., the perinatal phase of the offspring).

Important for consideration of these data is the fact that it was observed in two independently bred lines for high wheel-running behavior (S1 and S2). Numerous mechanisms may be involved in this phenotypic switch. However, when allowed one-week access to wheels (at week 6–8, and at 10 months of age) S1 and S2 mice did not show reductions in the number of daily wheel revolutions or average running speed due to the HE diet given perinatal and/or post-weaning (Fig. 4). Thus, we ruled out that HE diet exposure during the perinatal stage, during adulthood, or combinations of these caused S female mice to lose the trait for increased running wheel behavior. Effects of HE diet on wheel running behavior were only observed in CON females, with a clear increase in running activity by post-weaning HE diet. When viewing voluntary home cage activity (by passive infra-red detection) in mice that were not exposed to running wheels, this was reduced by HE diet only in CON mice, with a reduction by perinatal HE diet in males, and by post-weaning HE diet in females. In the S lines, however, none of the effect reached significance (Fig. 4). Although the trait for increased wheel-running behavior remained unaffected by combined perinatal and post-weaning HE exposure, this apparently did not block HE-induced weight gain. This loss of resistance to DIO by combined perinatal and post-weaning HE diet exposure in the S female was associated with clear disturbances in fuel homeostasis during oral glucose tolerance testing (OGTT) at 8 months of age (see Fig. 4).

Despite the similarities between S1 and S2 females, there were also some differences between the two S lines (see also [38]). First of all, reversal of DIO resistance towards a DIO-prone phenotype by perinatal HE diet exposure was clearly stronger in the S1 females than in the S2 females (although in a direct comparison between S1 and S2 this was not significant). Secondly, weight gain in the S1 female offspring was associated with OGTT-induced hyperinsulinemia - which is a read-out for insulin resistance [39] (Fig. 4), but with OGTT-induced hyperglycemia in S2 female offspring that was significantly stronger than in S1 females. Thirdly, as previously shown by us [26], S1 females are metabolically highly flexible; they increase metabolic rate (MR) and physical activity when given a high-fat diet in non-reproductive conditions and offset these responses under reproductive conditions. This compensatory increase of MR in S1 females when subjected to post-weaning HE diet was lost when their mothers were subjected to the HE diet, and showed

Fig. 4. Glucose homeostasis. Glucose (top panels A and B) and insulin (bottom panels C and D) responses to an oral glucose tolerance test (2 mg/kg) in male (left panels) and female (right panels) mice from the control (CON) and the two selected lines (S1, S2) at eight months of age. The area under the curve (AUC; expressed in mmol*L^-1*min^-1 for glucose, and in ng*min^-1*ml^-1 for insulin) was measured with the trapezoid method. Mice were subjected to combinations of low fat (LF) and high energy (HE) diet conditions during the perinatal and post-weaning stage. Open bars (LF-LF), closed bars (LF-HE), small dashed pattern (HE-LF), large dashed pattern (HE-HE), with the first abbreviation referring to the perinatal stage and the second to the post-weaning stage. Data are mean ± SEM. *perinatal HE, #post-weaning HE diet contrasts (p < 0.05) (for ANOVAs, see Results section).
an overall decrease in MR (both RMR, PA-MR) and a tendency to decrease home-cage activity. Contrary to S1 females, however, S2 females presented a further increased MR when experiencing the combined perinatal and post-weaning HE diets (Table 3).

As opposed to findings that S1 as well as S2 females fully resisted adulthood DIO following either perinatal or post-weaning HE-diet exposure, the data in the S males were less clear. In fact, the post-weaning HE diet exposure in offspring from LF feeding mothers (i.e., thus without the effects of HE programming) on DIO development were even stronger in S1 males than in the CON males. CON males of this Hsd:ICR outbred strain were previously reported to be rather insensitive to the obesogenic effects of a HE diet, as opposed to the commonly studied C57Bl/6J strain [40]. Thus, the S1 line appears to possess a male-dependent genotype that is able to switch phenotypic appearances depending on the type of diet. One mechanism potentially underlying this effect may be that it is linked to a sex-specific hyperactivity trait of S1 mice, as observed in these animals in the present study and in a previous study from this laboratory [21], with high levels of voluntary cage activity and wheel-running behavior in females, but relatively normal levels of these in males. In S2 mice, penetration of the “high-activity genotype” is found in females as well as in males. Because the above-mentioned sex-specific penetration in S1 mice is maintained despite continuous selection, it may be speculated that X or mitochondrial chromosomal loci in S1 mice play a role in this mechanism, which could be arrested by Y-chromosomal loci, such as the SRY gene [41]. For additional differences between lines S1 and S2, see Hannon et al. [42]. Despite these differences, however, all male offspring irrespective of line had markedly elevated OGTT-induced insulin responses only when subjected to the combined perinatal and post-weaning HE diet, indicating homologous programming and post-weaning effects of HE exposure on insulin signaling.

Increased adiposity in all offspring by perinatal HE diet was clearly evident at PND21, with an increase in gonadal fat pad mass strongly contributing to this effect (Fig. 1). This is in line with several other studies showing early nutritional effects in rodent offspring caused by dietary maternal manipulation [13, 43–46]. In a parallel study using the same lines and diets [37], milk energy output (MEO) was markedly lower in S dams compared to CON dams feeding the LF diet, but when subjected to a HE diet dams of both S lines dramatically caught up their MEO to even slightly increased levels above those found in the CON line upon exposure to a HE diet [37]. This may suggest that increased maternal milk energy transfer is involved in inducing weight gain in offspring, and this is apparently not hindered by the trait for high wheel-running behavior. In fact, we found that the increased body adiposity levels were associated with markedly higher plasma leptin levels in male and female offspring from both S lines compared to CON offspring (Fig. 1, A and C). Although we do not know the nature of this discrepancy, it is unlikely that increased adipocyte size alone underlies this effect given that congruency between leptin levels and adipocyte size was only observed in females, but not males (Fig. 1, panel D).

Increases in plasma leptin during the pre-pubertal phase are known to stimulate the reproductive axis and facilitate puberty onset [47] and has potent effects on skeletal maturation [48]. Although this was not directly assessed in the present study, it may be speculated that S offspring mature faster in general than CON offspring when subjected to a HE diet during the perinatal stage. The exact mechanisms underlying this hyperleptinemic response have to be resolved. It could for instance be speculated that specific local inflammatory pathways in fetal adipose tissue of S mice are involved in inducing these responses [49]. Increased leptin levels could then affect brain development [50] underlying a potential mechanism that could override activity-related DIO resistance resulting in obesity in S groups. While such a mechanism seems plausible, it apparently did not provoke major changes in ingestive behavior.

**Fig. 5.** Running wheel activity. Running wheel activity (expressed in revolutions/day) over a 5 day period in control (CON) and activity selected (S1 and S2) male (left panels A and C) and female (right panels B and D) mice at 6–8 weeks of age (top panels) and at 10 months of age (bottom panels). Mice were subjected to combinations of low fat (LF) and high energy (HE) diet conditions during the perinatal and post-weaning stage. Open bars (LF-LF), closed bars (LF-HE), small dashed pattern (HE-LF), large dashed pattern (HE-HE), with the first abbreviation referring to the perinatal stage and the second to the post-weaning stage. Data are mean ± SEM. *post-weaning HE diet contrasts (p < 0.05) (for ANOVAs, see Results section).
and/or physical voluntary activity later in life to underlie the phenotypic switch, at least under the conditions tested in the present study. Finally, the aforementioned lower MEO during lactation under LF conditions in the S lines that apparently co-evolved over the course of 50 generations of selection for high wheel-running behavior may in fact have been a multi-generational condition of relative under-nutrition, which recently was shown by Hardikar et al. to trigger weight gain and fuel disturbances in rats that were amplified by a HE diet exposure [45]. It would be of interest to investigate whether bi-directional relations exist between under-nutrition and hyperactivity, and whether continuous access to the running wheels from before conception onwards, as for example reviewed by Nathanielsz et al. [46], would have changed the outcome of our results. Although we anticipate a strong component of maternal programming in any of these possibilities, some of the offspring effects, at least in our experiments, may even have been produced through paternal programming [51], since the males that were paired with the females to produce the offspring ate from the same diet (LF or HE diets) as the females did.

5. Conclusions and perspectives

In summary, resistance to HE DIO in adult female mice from lines selectively bred over ~50 generations for increased wheel running behavior was blocked by additional perinatal HE diet exposure in only one cycle of breeding. An explanation for this effect is that potential allelic variants underlying the trait of DIO proneness were not eliminated but rather silenced by the selection protocol, and switched on again by perinatal HE diet exposure by epigenetic mechanisms [52, 53]. From a biological standpoint, the above-mentioned phenomenon could represent an example of an adaptive mechanism (at least under natural conditions) by which an elevation of the plasma leptin level in the adolescent state advances maturation and therefore could reduce the age at first reproduction in the face of food abundance. Thus, we speculate that the co-segregation of hyperleptinemia at weaning following nutrient excess with the trait for high running wheel behavior (which was notably observed in both independently selected lines) could be explained as an advantageous linkage of traits that have benefitted survival of species shifting between episodes of food shortage and food abundance. Such individuals would be clearly able to increase their habitat (via locomotor activity) in the face of normal or shrinking food reserves, while during food abundance they would advance fecundity (via a leptin mediated pathway). According to West-Eberhard, developmental switches such as observed in the S mice may be seen as decision points in the ontology of behavior, physiology, and morphology and contribute to the modularity and dissociability of the phenotypic subunits we call “traits” [54]. When recombined, these traits may have an increased fitness value from an evolutionary perspective, and may improve survival of successive generations [31, 54–56]. It would be of interest to investigate whether interaction between traits for voluntary activity and thrift are also relevant for the rapid spread of human obesity over the last few decades, with the cardio-metabolic disease epidemic as overpowering pleiotropic side effect impeding general sustainable health [57–60].

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

SG and NM collected the data. SG and GvD designed the study, analyzed the data and wrote the manuscript. TG and AJWS and MCH reviewed the manuscript. TG provided the mouse strains. EP and MCH helped with adipose tissue histology. All authors read and approved the final manuscript.

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