Effects of laboratory housing conditions on neurobiology of energy balance in mice

Karapetsas, Giorgio

DOI:
10.33612/diss.182828078

Chapter 4

Post-weaning individual housing of C57BL/6J female mice does not affect energy balance compared to social housing, but social status does

Giorgio Karapetsas[1], Steffen van Heijningen[1], Jan Komdeur[1], Lidewij Schipper[1,2], Gertjan van Dijk[1]

[1] GELIFES, Groningen Institute for Evolutionary Life Sciences, University of Groningen, The Netherlands

[2] Danone Nutricia Research, Utrecht, The Netherlands

Abstract

In mice, postweaning individual versus social housing can have profound effects on energy balance regulation and growth. However, the effects of postweaning individual versus social housing have been investigated mainly in male subjects. The primary aim of the present study was to investigate the effects of postweaning individual (IND, 1 individual/cage) versus social (SOC, 2 individuals/cage) housing parameters for growth, energy balance regulation and metabolic health status in female C57BL/6J mice, under normal (LF) and high fat diet (HF) conditions. Body weight was monitored throughout the experiment, food intake was assessed at postnatal day (PND) 92-97 and bone mineral content and bone mineral density were evaluated with dual energy absorptiometry (DXA) at PND97. Body composition was determined at PND126 with fat extraction using a soxhlet apparatus, when plasma and hypothalamic slices were collected for further analyses. Since social rank might contribute to some of the variation in phenotypical outcomes observed in SOC housed females, an exploratory analysis was conducted by the use of a tube test at PND 124-125 in SOC housed females only. Diet, but not housing, affected body weight trajectories and adiposity in female mice. The effects of IND on energy balance regulation were subtle, and were characterized by increased food intake and lean mass compared to SOC. Reanalysis of these parameters according to dominance hierarchy showed that dominant female mice had higher weight gain relative to subordinate ones, only in HF diet conditions. Dominant females showed an increase in lean mass and fat mass and an increase in plasmatic leptin levels irrespective of diet type. The findings of the current study show that differences in energy balance regulation between SOC housed female littermates and IND housed ones are relatively small, in comparison to the impact of feeding a HF diet versus a LF diet and that social rank may lead to changes in energy balance regulation. These findings may contribute to explain some of the variation within and between studies and help select appropriate housing conditions in future studies.

Introduction

Mice are extensively used in disease-related research. Because mice are social animals with specific needs from the environment, laboratory and housing protocols can have profound effects on the physiology of this species (Schipper et al., 2018; Kappel, Hawkins, and Mendl 2017). Although a social (SOC) housing condition is obviously best to suit social needs (Council 2011; Van Loo, Van Zutphen & Baumas, 2003), individual (IND) housing is frequently applied to allow for the presence of exteriorized devices (Kappel, Hawkins, and Mendl 2017), to prevent fighting among cagemates (Zidar et al., 2019) and to better differentiate among animals when measuring individual parameters such as food intake and energy expenditure (Tschöp et al. 2012). Apart from the affective consequences of IND versus SOC housing of laboratory mice (Võikar et al., 2005), individual housing versus social housing may have considerable effects on energy balance regulation and may influence metabolic phenotype, especially when individual housing is applied early in life (Schipper et al. 2018). Indeed, we recently showed that individual (IND) compared to SOC (pair-wise) housing of male C57BL/6J mice at standard room temperature (RT; 21°C) from weaning age onwards reduces (adolescent) growth rate, followed by increased body weight gain and predisposition for obesity in adulthood (Schipper et al. 2020). Likewise, these findings were confirmed in chapter 3 of this thesis. The majority of published studies that focused on the effects of SOC housing conditions on energy balance regulation in rodents included males (Schipper et al. 2018). The limited number of studies on female rodents comparing the effects of postweaning (i.e. at 3 or 4 weeks of age) IND versus SOC housing on body weight trajectories showed contradicting results. Some studies demonstrated increased body weights as a result of post-weaning IND housing (Jahng et al. 2012; Weltman, Sachler, and Sparber 1966) while others reported no effects (Barnhart and Pizzi 1982; Chvédoff et al. 1980; Lopez and Laber 2015), or even a reduction due to individual housing (Guo et al. 2004; Wiberg, Airth, and Grice 1966). Most studies reporting effects of IND versus SOC housing on food intake in female rodents indicate that this parameter is increased by IND housing (Chvédoff et al. 1980; Jahng et al. 2012; Weltman, Sachler, and Sparber 1966; Morgan and Einon 1975), although one study showed that food intake was increased only in response to high fat feeding (Krolow et al. 2013). These data suggest that IND housing increases energy intake in female mice, however sufficiently detailed data on the effect of IND versus SOC housing on body composition and parameters linked to (neuro) endocrine regulation of energy metabolism is lacking. The primary aim of the present study was to investigate the effects of IND (1 indivdual/cage) versus SOC (2 individuals /cage) housing of female C57BL/6J mice on growth, body composition

and for parameters energy balance regulation from weaning till PND 126. Moreover, to investigate potential interactions between housing condition and diet on the aforementioned parameters we exposed subgroups of IND and SOC mice to either a healthy low fat (LF) maintenance diet, or a palatable high fat (HF) diet supplemented with sucrose from PND 42 onwards.

Mice form complex social hierarchies both in the wild and in laboratory settings (Wang, Kessels & Hu, 2014) and this might play a role in regulating energy balance (Tamashiro et al, 2004). Changes in body size among individuals living in the same group may be the result of developmental plasticity adjusting the offspring phenotypes to match the environment they experience and increase the chances of future survival. For example, it has been shown that body size can be affected by hierarchical status in male rats (Blanchard et al. 1995; Tamashiro et al. 2004). A common pattern in dominance hierarchies is that animals show different levels of psychosocial stress (Dadomo et al. 2011) and this may impact eating patterns and weight gain depending on social rank (Moles et al. 2006). Again, in rodents the majority of the studies investigating the effects of social dominance on energy balance have been focused on males. Generally, female rats are thought not to form strong social hierarchies (Tamashiro et al. 2004). Female mice, however, do show strong directionally consistent social relationships when housed in large groups, with hierarchies that are less linear and less despotic compared to male mice (Williamson et al., 2019). However, data on the formation of social hierarchies in pair-housed female mice is lacking. Interestingly, dominancy in non-human female primates showed that social hierarchies affect energy balance and metabolic health in female subjects. Indeed, dominant female primates showed increased body weight relative to subordinate (Wilson et al. 2008; Michopoulos and Wilson 2011) as well as increased fat mass, bone mass and higher circulating leptin levels (Michopoulos et al. 2012; Jarrell et al. 2008; Collura, Hoffman, and Wilson 2009). Therefore, in the assessment of SOC versus IND housing effects on energy balance and neuroendocrine regulation of metabolic parameters in female mice, the role of social rank might play a role in explaining some of the variation observed in SOC housed females. Therefore, in an exploratory analysis we assessed the potential interplay of social hierarchies in explaining the variation in energy balance regulation and metabolic health in the SOC group only.

Abbreviations

bone mineral content (BMC), bone mineral density (BMD), dual energy x-ray absorptiometry (DXA), high-fat (HF), individual housing (IND), postnatal-day (PND), low fat (LF), social (pair) housing (SOC)

Material and methods

Ethical statement

The animal experiment and procedures complied with the principles of laboratory animal care following the EU-directive for the protection of animals used for scientific purposes. Experimental procedures were approved by the Dutch Central Committee on Animal Experimentation (license number AVD1050020185905). C57BL/6J mice were selected for this study as they are prone to develop obesity and metabolic alterations (Collins et al., 2004) and has been already used by our laboratory, for example to investigate the effects of postweaning individual housing versus social housing on energy balance regulation in male C57BL/6J mice (Schipper et al. 2020).

Animals

Experimental groups were bred in house and obtained from C57BL/6J males and dams purchased from Charles River Laboratories (Sulzfeld, Germany). After two-weeks acclimation, one dam was introduced to a cage with a male for three days. After mating, dams were pair-housed for two weeks and singly housed for the last week of gestation. At postnatal day (PND) 2, litters were randomized and culled to 6 pups (male:female ratio of 3:3 or 4:2) to standardize litter size and to reduce litter-variability. Litter weight was measured once a week during lactation. In total, 48 female C57BL/6J experimental mice were obtained from eight different breeding batches that were performed once a week. Dams were fed a standard rodent diet (low fat diet - LF; Altromin® 1410 – 10 mm pellets) and water *ad libitum*. A full overview of the experimental outline is given in figure 1.

Housing conditions

All mice were weaned at PND 21 and housed in polycarbonate type II cages with bedding (Aspen wood shavings) and a plastic shelter (Red house; Techniplast, Va, Italy). Female offspring were randomly housed either individually (IND) or socially with a littermate (SOC, 2 animals per cage) and fed the same maternal LF diet and water *ad libitum* (n = 24 per group). Climate rooms were used to keep temperature and humidity constant (21 ± 1°C; 50 ± 5% humidity), and light/dark periods of 12hrs were maintained (with dark starting at 21:00 and light at 09:00).

A

	Test, procedure, event	Post-natal-day (PND)																					
		Adolescence							Adulthood														
		0	1	7	14	21	28	35	42	49	56	70	84	91	92	93	94	95	96	97	112	124	125	126
1	Birth																							
2	Litter randomization																							
3	Litter weight																							
4	Weaning																							
5	Housing assignment																							
6	Diet assignment																							
7	Body weight measurement																							
8	Automated food intake registration (TSE)																							
9	DXA																							
10	Tube test																							
11	Semi-fasting																							
12	Body composition analysis																							
13	Hypothalamic gene expression																							

B

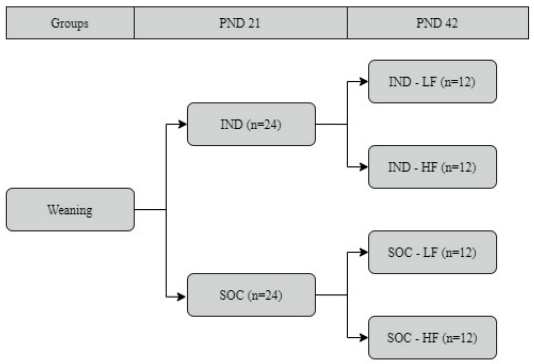

Figure 1. Timeline (A) and group overview (B) of the experiment. PND = postnatal day, IND = individual housing, SOC = social housing (pairwise), LF = low fat diet, HF = high fat diet.

Diets

At PND 42, after three weeks from the housing assignment, half of the groups were fed a high fat (HF) diet supplemented with sucrose until the end of the study. This gave rise to 4 groups: individually housed fed a low fat diet (IND–LF), socially housed fed a low fat diet (SOC–LF), individually housed fed a high fat/sucrose diet (IND–HF), socially housed fed a high fat/sucrose diet (SOC– HF). Each group consisted of 12 animals. Animals remained in their respective housing conditions and diet allocated until sacrifice at the end of the experiment (PND126). Researchers were aware of the group allocation, as housing and diet could not be blinded, but were not aware of the dominance relationships. The HF diet was made in-house by using grounded Altromin® 1410 (46.5% of total weight) as base, with addition of lard (14.5%), soy oil (4.7%), sucrose (17.4%), arabic gum (2.3%), casein (10.5%), mineral/vitamin mix (respect. 2.3% and 1.7 % added to compensate for dilution of the LF diet base from which the HF diet was produced) and 10% water.

After mixing for 1 hr, the dough was used to make pellets which were dried, and subsequently frozen in air tight bags at -18°C, until use. Because fats in the HF diet become rancid, the HF diet in food hoppers was replaced completely once a week. The HF diet was replaced once a week following two hours of thawing. On the other hand, the LF was kept at RT (21°C) and refilled once a week. Both diets were provided by a food hopper. The composition of the experimental diets is represented in table 1.

	Low fat diet (LF)	High fat + high sugar diet (HF)
Fat (w/w%)	9,1%	25,6%
Energy from fat (%)	22%	44,7%
Carbohydrates (w/w%)	47,4%	24,3%
Energy from Carbohydrates (%)	50%	35,7%
Proteins (w/w%)	25,3%	24,3%
Energy from proteins (%)	28%	19,6%
Total energy	**3,68 Kcal/Kg**	**4,77 Kcal/Kg**

Table 1. Weight and energy content of the experimental diets.

Body weight measurements

Body weight (BW) was recorded weekly from PND21 until PND56 and at PND70, PN84, PND91, PND97, PND112, PND125 and PND126.

Automated food intake registration

From PND 91 to PND 97, food intake was monitored using a feeding/drinking automated apparatus from TSE-System (Feeding and drinking, Bad Homburg vor der Höhe, Germany). However, only food intake (and not drinking behaviour) was assessed to the nearest 0.04g every 10 seconds over a period of 6 days. To avoid the stress of placing the mice in a metabolic chamber, the lid of the mice' home cages was adapted to fit the system's food hopper (Feeding sensor advanced, 259998-SEN/FED). The first day of food registration was excluded from the calculations to avoid variation due to the habituation to the novel food hopper and only the last 5 days were considered for food intake analyses. For socially housed animals, total food intake was divided by two and only one statistical unit was used for the pair. Food intake was expressed either as daily food intake in kilojoules (KJ) or daily food intake corrected for body weight (KJ / g BW), therefore for each animal or pair, a total of five observations were present and 5 units were used (IND: 24 x 5 = 60, SOC: 12 x 5 = 30). For socially housed mice, the averaged BW of the pairs was used.

Dual energy x-ray absorptiometry (DXA)

Right after the end of automated food intake registration, at PND 97, bone homeostasis was analyzed by dual energy x-ray absorptiometry (DXA) with a pDEXA apparatus from Norland Stratec. The mouse was anaesthetized under light isoflurane anesthesia in an induction chamber (flow meter set to 0,8L/min and 4-5% isoflurane) and then placed in the DXA apparatus where the anesthesia was maintained with a tube positioned on the mouse nose (flow meter set to 0,5L/min and 1-8%-2,2% isoflurane). Spontaneous breathing pattern was constantly monitored during the test by an operator. After the test (duration 12-15 minutes), the mouse was removed from the apparatus and let recover alone in a cage provided with a heating mat for 10-15 minutes, until complete awakening was re-established. After complete awakening, the mouse was reintroduced in its home-cage with (SOC) or without cage-mate (IND). Bone mineral content (BMC) is reported in grams and bone mineral density (BMD) in grams/cm^2.

Tube test

Social hierarchy was evaluated by the means of a tube test performed at PND124-125 in the SOC group only. Two different tubes were used: one for HF-fed mice with an inside diameter of 33mm and one for LF-fed mice (standard) with an inside diameter of 30mm. This was necessary due to the differences in body size between LF and HF mice. Both the tubes were 30cm long. Briefly, on the first day the mice were habituated and trained to the tube. The red plastic shelter was removed and the tube was placed in the home cage of the mice. After one minute of habituation, one mouse at a time was gently guided inside the tube and let it successfully walk through for a total of ten times. The second day, the pair-housed mice were tested for a total of five times successively in order to obtain dominance status (winner versus loser). The mouse able to push the other mouse out of the tube was considered the winner. While both mice were inserted simultaneously into the tube, a sliding door in the middle of the tube was removed allowing the passage of the mice. The mouse rank (1 to 5) was assessed by the number of wins a mouse won against its cage mate. The mouse that won more trials was considered the dominant, while the losing mouse was considered the subordinate.

Tissue collection and body composition analysis

To induce a semi-fasting state at sacrifice (PND126), mice were weighed at the previous day (5 pm) and were provided with half the amount of their normal nocturnal food intake calculated with TSE system (PND91-97), by taking into account the food eaten for three consecutive evenings/nights. Animals were sacrificed between 9 am and 12 pm. Mice were anesthetized by inhalation of isoflurane (4-5 % and flow meter set to 0,8L/min) and subsequently heart puncture was performed for blood collection followed by decapitation. Blood was collected in EDTA tubes maintained at melting ice, centrifuged at 2600G for 10 minutes, and plasma then taken into sealing tubes and stored at -80°C until further analyses. Brain regions (hypothalamus) and fat pads (perirenal, retroperitoneal, inguinal, subcutaneous, brown adipose tissue) were dissected, weighed and snap frozen and stored at -80°C. The left femur was dissected and its length was measured using a digital micro-caliper. Carcasses were frozen at -20°C till they were placed at 60°C for two weeks until complete drying. Fat extraction was then performed with petroleum ether in a soxhlet apparatus as previously described (Reijne et al. 2016). Total fat mass (FM) and total lean mass (LM) were given in grams, and the percentage of total fat mass (%FM) was calculated by the following formula [(total fat mass / body weight) x 100]. The percentage of body lean mass (%LM) was calculated by the following formula [(body lean mass / body weight) x 100]. The fat mass to lean mass ratio (FM/LM) was calculated by the following formula (total fat mass / body lean mass).

Plasma measurements

Plasma corticosterone (CORT) and insulin were analyzed in duplicate by commercial RIA kits (CORT: MP Biomedicals, Orangeburg, NY, cat. No 07-1201103; insulin: Millipore, St. Charles, Missouri, cat. No #RI-13K). Plasma glucose levels were assessed by the ferricyanide method by Hoffman (Hoffman 1937). HOMA-IR was obtained with the following formula (*[glucose]*[insulin]/14.1*) as reported previously (van Dijk et al. 2013). Plasma triglyceride content was determined with a commercial kit (Roche DIagnostic, Mannheim, Germany; C.f.a.s calibrator Nr: 10759350 & Cobas Triglyceride Nr. 20767107322). Plasma leptin concentrations were determined with a commercial kit (Millipore). Four samples for insulin and CORT, three for glucose, five for triglyceride and one for leptin could not be analysed due to unforeseen circumstances.

RNA isolation and quantitative real-time PCR

Hypothalamic gene expression analysis was performed to study genes regulating energy balance regulation and growth. Hypothalamic RNA was extracted using NucleoSpin® kit (Macherey-Nagel) according to manufacturer's instructions and it was used as template for cDNA synthesis using iScript cDNA synthesis kit (Biorad®). A Nanodrop spectrophotometer 2000c assessed the quality of the RNA. Quality was considered acceptable when the A260/A280 ratio was > 1-8. RNA expression of the genes of interest was measured using real time polymerase chain reaction (RT-PCR) using SYBR Green (Thermofisher®). Forward and reverse

primers for *Bdnf*, *Pomc*, *Npy*, *Lepr*, *Mc4r*, *Crh*, *Ghrh*, *Somatostatin*, *Socs3*, *Ptpn1*, *Ikk-β* and housekeeping gene *Gapdh* are shown in table 2. The lay-out for the PCR plates were designed to minimize between-plate variances. Furthermore, primer concentrations were optimized resulting in the following final concentrations (in nM) for forward and reverse primers, respectively: *Bdnf* (520:380); *Pomc* (520:520); *Npy* (520:520); *Lepr* (380:520); *Mc4r* (380:520); *Crh* (400:400), *Ghrh* (400:400), *Somatostatin* (400:400), *Socs3* (400:400), *Ptp1b* (400:400), *Ikk-β* (400:400) and *Gapdh* (240:240). In each well 1 ml of 5 ng/L cDNA, 5 uL SYBR green, 1 uL forward primer, 1 uL reverse primer and 2 uL water was used. The samples were run at the following program: 95°C for 2 min; 50x (95°C for 3 seconds, 60°C for 30 seconds); melting temperature protocol (60°C to 95°C, increment 0.2°C for 5 second). Triplicates were made for each gene per sample. The relative gene expression was calculated using the delta delta Ct method using *Gapdh* as housekeeping gene. Non-baseline corrected data was processed and corrected using LinregPCR (version 2018.0) to determine the PCR efficiency for each sample, the efficiency per amplicon group and for Cq determination. Samples with either deviating individual PCR efficiency of more than 5%, or baseline errors or noisy samples have been excluded from the calculations.

Gene	Forward primer	Reverse primer
Bdnf	GGTATCCAAAGGCCAACTGA	GCAGCCTTCCTTGGTGTAAC
Pomc	ACCTCACCACGGAGAGCA	GCGAGAGGTCGAGTTTGC
Npy	ATGCTAGGTAACAAGCGAATGG	TGTCGCAGAGCGGAGTAGTAT
Lepr	CCTCTTGTGTCCTACTGCTCG	GAAATTCAGTCCTTGTGCCCAG
Mc4r	CCCGGACGGAGGATGCTAT	TCGCCACGATCACTAGAATGT
Crh	CCTGGGGAATCTCAACAGAA	AACACGCGGAAAAAGTTAGC
Ghrh	TGCCATCTTCACCACCAAC	TCATCTGCTTGTCCTCTGTCC
Somatostatin	TCTGCATCGTCCTGGCTTT	CTTGGCCAGTTCCTGTTTCC
Socs3	CACCTGGACTCCTATGAGAAAGTG	GAGCATCATACTGATCCAGGAACT
Ptp1b	GCGCTTCTCCTACCTGGCTGTCAT	ACGTGCTCGGGTGGAAGGTCTA
Ikk-β	CGGCCCTTCCTCCCTAAC	GGTGCCACATAAGCATCAGC
Gapdh	ACAACTTTGGCATTGTGGAA	GATGCAGGGATGATGTTCTG

Table 2. Overview of forward and reverse primers used for RT-PCR.

Statistical analyses

Sample size was estimated based on our previous experience in carrying out metabolic experiments involving (male) C57BL/6J mice. Overall, sample size was calculated taking into account the number of animals necessary for a larger experiment in which the effects of IND versus SOC on energy balance regulation in male and female mice. An average effect size of 0.29, an α error prob. of 0.05 and a power of 0.8 for a total of 24 through an F-test (ANCOVA, fixed effects, main effects and interactions) provided a minimal group size of 12 animals per group.

Effects of housing and diet

Statistical analyses were performed using RStudio 1.3.959 (R Core Team, 2013). A general linear mixed-effects model was used to examine whether housing predicted body weight in adolescence (from PND21 to PND42) (lmer in the lme4 R package), which adjusted for the repeated measures and with a random intercept for mouse identity. Each group included 24 mice (IND, SOC; n = 24, figure 1). A second general linear mixed-effects model was used to investigate whether housing and diet predicted body weight from PND49 to PND125 (adulthood). The body weight on the morning of the sacrifice (PND126) of the animals was not included in this model, as the animals were fasted, but it was used for\ calculating %FM and %LM at PND126. General linear models were constructed to evaluate the effects of the factors housing and diet on body weight, BMC, BMD, body composition parameters (FM, LM, FM/LM, FM%, LM%, femur length, fat pads weight), plasma measurements and hypothalamic relative gene expression. Each group included a total of 12 animals, unless specified otherwise (IND-LF, SOC-LF, IND-HF, SOC-HF; figure 1). A general linear mixed-effects model was used to examine whether housing and diet predicted daily food intake (lmer in the lme4 R package), which adjusted for the repeated measures with a random intercept for mouse identity. In these models, we also examined whether potential interactions between factors were present. Results were interpreted after homogeneity and normality were assessed. Post hoc comparisons using Tukey's method were performed to assess significant relationship between groups (emmeans R package).

Exploratory analysis into the effects of hierarchy and diet (only in socially housed mice)

Using data only from socially housed animals, a general linear mixed-effects model was used to examine whether social hierarchy predicted body weight in adolescence (from PND21 to PND42) (lmer in the lme4 R package), which adjusted for the repeated measures and with a random intercept for mouse identity. A second general linear mixed-effects model was used to investigate whether social hierarchy and diet predicted body weight from PND49 to PND125 (adulthood). General linear models were constructed to evaluate the effects of the factors social hierarchy and diet on body weight, BMC, BMD, body composition

parameters (FM, LM, FM/LM, FM%, LM%, femur length, fat pads weight), plasma measurements and hypothalamic relative gene expression. In these models, we also examined whether potential interactions between factors were present. Results were interpreted after homogeneity and normality were assessed. Post hoc comparisons using Tukey's method were performed to assess significant relationship between groups (emmeans R package). In this exploratory analysis, 4 groups were included: dominant–LF n=4, subordinate–LF n=4, dominant–HF n=6, subordinate– HF n=6. Four mice were not tested in the tube test due to unforeseen circumstances, therefore the dominant–LF and subordinate–LF groups have 2 statistical units less than the HF groups.

Graphical design was performed using the ggplot2 R package. All data is presented as mean ± SD, except for the qPCR data which are presented as mean relative expression with 95% confidence intervals. Data are considered significantly different when $p < 0.05$.

Results

Effects of post-weaning IND versus SOC housing

Body weight

Body weight did not differ at the beginning of the housing assignment between groups (PND 21) ($p = 0.47$). Housing conditions did not affect adolescent growth (between PND21 and PND42, p=0.67, figure 2A) and adult body weight over time period (between PND42 and PND125, p= 0.99, figure 2B). Exposure to HF diet during adulthood increased body weight ($p < 0.001$, figure 2B) relative to the LF diet condition.

Food intake

Daily food intake was measured during adulthood. IND housed mice showed higher energy intake than SOC housed mice, both when this was expressed as KJ ($p = 0.02$, figure 3A) and corrected per BW ($p = 0.02$, figure 3B). Food intake expressed as KJ was increased in HF fed mice compared to LF feeding ($p = 0.001$, 3A), but this difference became non-significant after correcting for body weight (figure 3B).

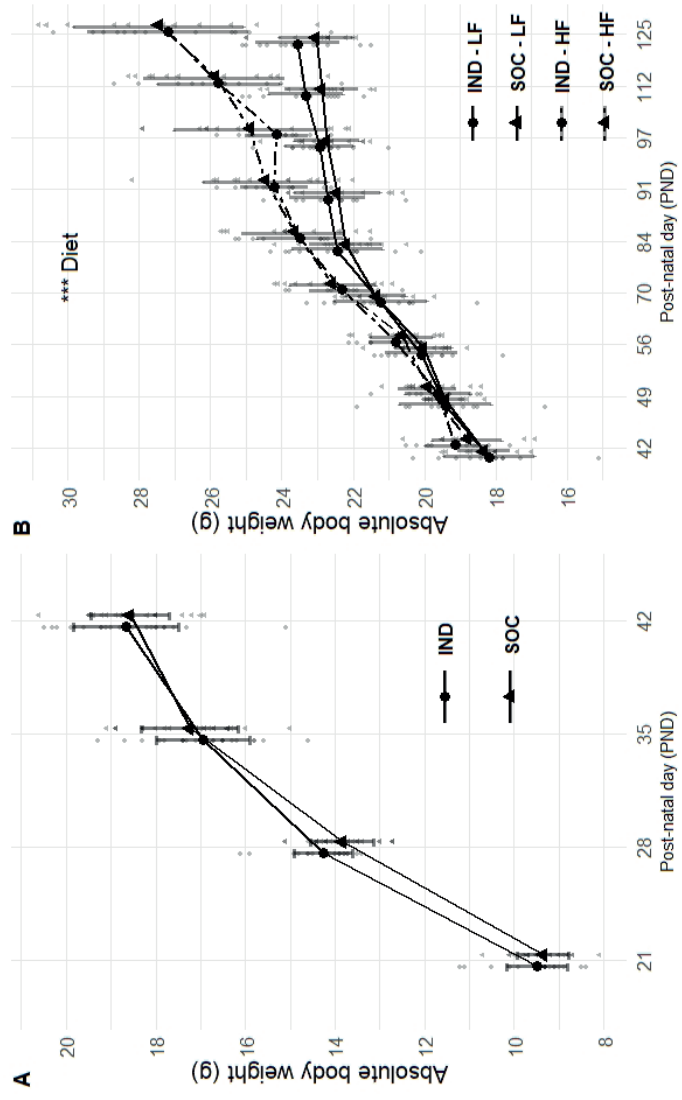

Figure 2. Effect of postweaning IND versus SOC and/or a LF versus HF on body weight. (**A**) Absolute body weight (g) of IND and SOC female mice from weaning (PND21) till PND42 (n=24 per group). (**B**) Absolute body weight (g) of IND and SOC mice fed either a LF or a HF from PND42 till PND125 (n = 12 per group). Data are expressed as individual points and means ± standard deviation (SD). P-values are expressed as * (< 0.05), ** (< 0.01) and *** (< 0.001).

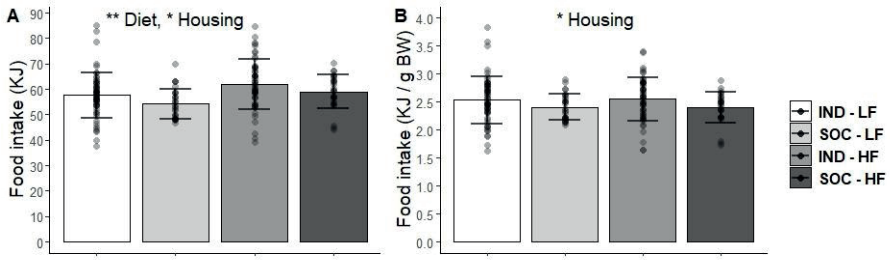

Figure 3. Average daily food intake assessed in the automated feeding/drinking TSE system. (A) Daily food intake expressed in KJ. (B) Daily food intake corrected per gram of body weight. (few data points were excluded due to technical issues: IND–LF n=59, SOC–LF n=29, IND–HF n=53, SOC– HF n=28). Data is expressed as individual points and means ± standard deviation (SD). P-values are expressed as * (< 0.05), ** (< 0.01) and *** (< 0.001).

Bone mineral content and bone mineral density

At PND97, bone mineral content (BMC) was unaffected by housing ($p = 0.86$) and significantly increased by high fat feeding ($p = 0.03$) (figure 4A). A significant housing x diet interaction was present for bone mineral density (BMD) ($p < 0.001$) (figure 4B). Tukey post-hoc analysis showed that BMD was significantly increased in the SOC-HF group compared to the other three groups ($p < 0.001$, 4B).

Body composition

At PN 126**,** IND housed animals showed increased lean mass compared to SOC housed animals ($p = 0.01$, figure 5D), however other parameters related to body composition remained unaffected by housing conditions (figure 5 and table 3). HF diet feeding resulted in higher body weight ($p < 0.001$, figure 5A), fat mass ($p < 0.001$, figure 5B), fat mass (%) ($p < 0.001$, figure 5C), lean mass ($p = 0.03$, figure 5D), fat to lean mass ratio ($p < 0.001$, figure 5E), weights of white and brown adipose tissue depots ($p < 0.001$, table 3) and femur length ($p = 0.01$, figure 5F).

Hypothalamic gene expression

While IND housing significantly decreased *pomc* hypothalamic expression ($p = 0.04$) the expression of *npy*, *mc4r*, *bdnf*, *crh*, *ghrh*, *somatostatin*, *Socs3*, *Ptp1b* and *Ikk-β* was not different compared to socially housed animals. HF fat diet feeding increased *npy* expression ($p = 0.008$) and a diet x housing interaction was present for hypothalamic *lepr* ($p = 0.001$). Tukey post-hoc analysis showed that SOC housing significantly increased *lepr* expression compared to IND in HF ($p = 0.001$) but not in LF-groups. Other genes investigated were not affected by HF feeding.

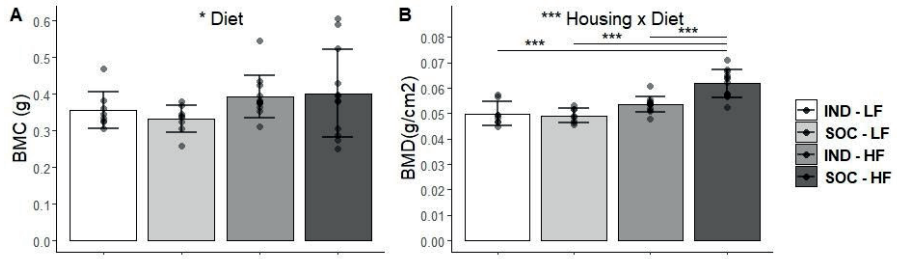

Figure 4. (A) Bone mineral content and (B) bone mineral density assessed by dual energy x-ray absorptiometry (DXA). (IND–LF n=8, SOC–LF n=8, IND–HF n=12, SOC– HF n=12). Data is expressed as individual points and means ± standard deviation (SD). P-values are expressed as * (< 0.05), ** (< 0.01) and *** (< 0.001).

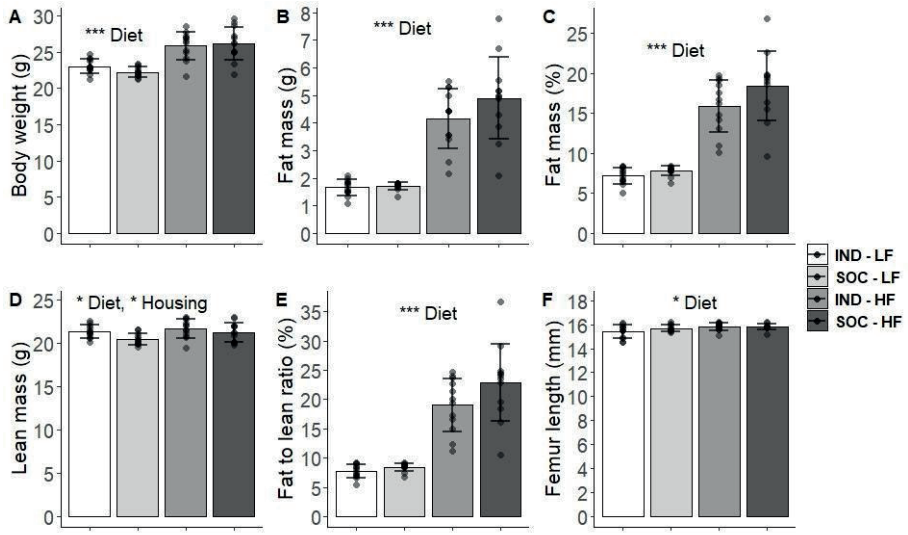

Figure 5. Body composition analysis assessed at PND126 by fat extraction performed with petroleum ether in a soxhlet apparatus. (**A**) Fat mass (g), (**B**) fat mass (%), (**C**) lean mass (g), (**D**) fat to lean mass ratio, (**E**) femur length. Data are expressed as individual data points, means ± standard deviation (SD). n=12 per group. P-values are expressed as * (< 0.05), ** (< 0.01) and *** (< 0.001).

	IND - LF	SOC - LF	IND - HF	SOC - HF	main effect
WAT, perirenal (mg)	61.5 ± 26.8	72.2 ± 33.4	142.4 ± 83.3	167.9 ± 126.2	diet ***
WAT, retroperitoneal (mg)	38.4 ± 12.3	43.5 ± 12.7	146.1 ± 67.5	184.5 ± 93.8	diet ***
WAT, inguinal (mg)	182.7 ± 48	194.3 ± 65.8	482.9 ± 144.1	588.7 ± 216.8	diet ***
WAT, subcutaneous (mg)	478.3 ± 90.1	483.1 ± 68.5	1128.3 ± 326.8	1266.5 ± 351.8	diet ***
WAT, muscle & organs fat (mg)	764.6 ± 161.9	794.3 ± 120.6	2014.2 ± 571.9	2404.2 ± 790.8	diet ***
BAT, interscapular (mg)	134.5 ± 15.8	135.1 ± 23.7	231.9 ± 58.4	284.1 ± 75.7	diet ***

Table 3. Fat pads weight assessed at PND126 by carcass analysis and fat extraction performed with petroleum ether in a soxhlet apparatus. Data is expressed as means ± standard deviation (SD). n=12 per group. P-values are expressed as * (< 0.05), ** (< 0.01) and *** (< 0.001).

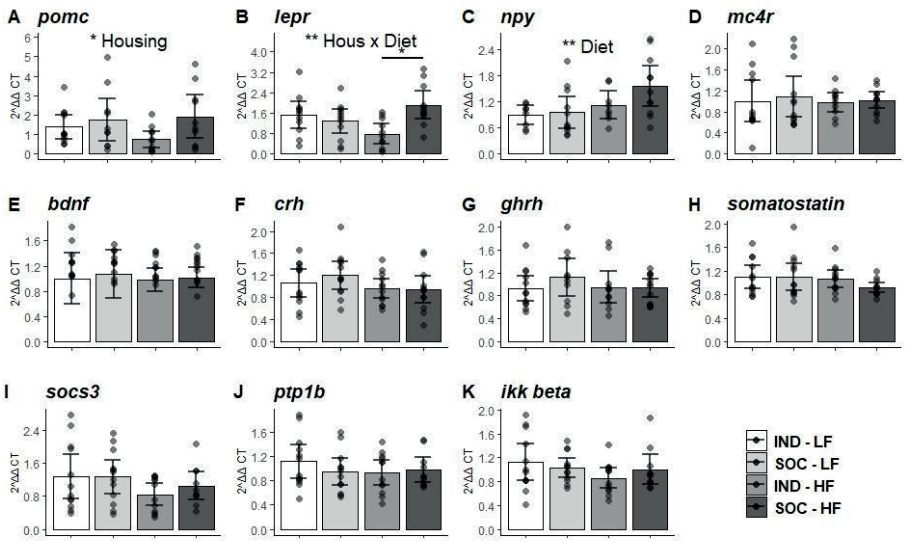

Figure 6. Relative *pomc*, *lepr*, *npy*, *mc4r*, *bdnf*, *crh*, *ghrh*, *somatostatin*, *socs3*, *ptp1b* and *ikk-β* hypothalamic expression. Data is expressed as individual units, means ± 95% confidence intervals. (*pomc*: IND–LF n=11, SOC–LF n=10, IND–HF n=11, SOC– HF n=10 – *lepr*: IND–LF n=11, SOC–LF n=11, IND–HF n=11, SOC– HF n=11 – *npy*: IND–LF n=8, SOC–LF n=11, IND–HF n=8, SOC– HF n=11 – *mc4r*: IND–LF n=11, SOC–LF n=12, IND–HF n=10, SOC– HF n=11 – *bdnf*: IND–LF n=11, SOC–LF n=11, IND–HF n=11, SOC– HF n=12 – *crh*: IND–LF n=12, SOC–LF n=12, IND–HF n=12, SOC– HF n=12 – *somatostatin*: IND–LF n=12, SOC–LF n=12, IND–HF n=12, SOC– HF n=12 – *ghrh*: IND–LF n=12, SOC–LF n=10, IND–HF n=11, SOC– HF n=12, *Socs3, Ptp1b* and *Ikk-β*: IND–LF n=12, SOC–LF n=12, IND–HF n=12, SOC– HF n=10). P-values are expressed as * (< 0.05), ** (< 0.01) and *** (< 0.001).

Plasma levels of glucose, insulin, corticosterone, HOMA-IR, triglycerides and leptin

Plasma glucose, insulin, calculated HOMA-IR and triglyceride were unaffected by diet and housing (figure 7A-C). On the other hand, a housing x diet interaction was present for plasma corticosterone (p = 0.047). Tukey post-hoc test showed that plasma CORT was increased in the SOC-LF group compared to IND-LF (p = 0.002), while this difference was not present in the HF groups (figure 7D). Leptin levels were significantly increased by HF-feeding compared to LF-feeding (p < 0.001), irrespective of housing conditions (figure 7F).

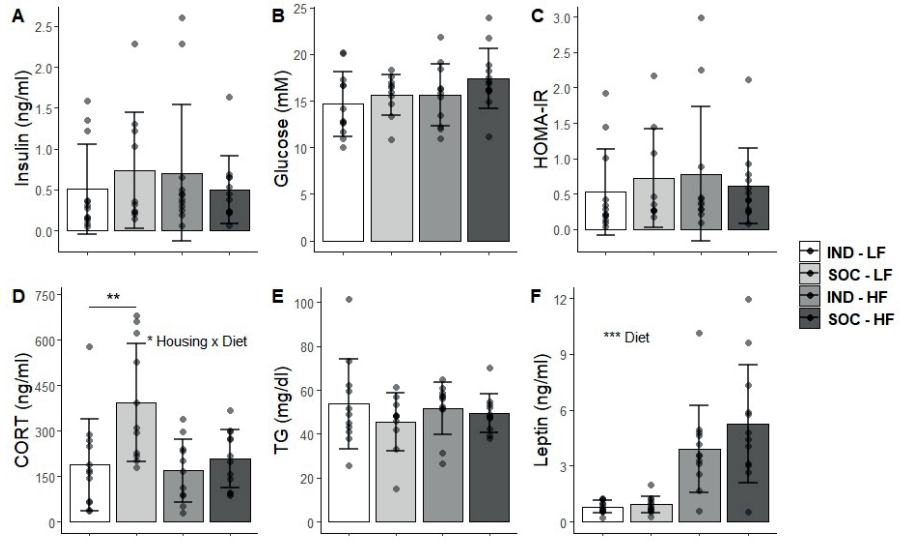

Figure 7. Plasma levels of **(A)** insulin (ng/ml), **(B)** glucose (mM), **(C)** calculated HOMA-IR **(D)** plasma corticosterone (ng/ml), (**E**) triglyceride (mg/dl) and (**F**) leptin (ng/ml). Group composition is as follow: *insulin* IND–LF n=12, SOC–LF n=10, IND–HF n=12, SOC– HF n=12; *glucose* IND–LF n=12, SOC–LF n=10, IND–HF n=11, SOC– HF n=12; *HOMA-IR* IND–LF n=12, SOC–LF n=9, IND–HF n=11, SOC– HF n=12; *CORT* IND–LF n=12, SOC–LF n=11, IND–HF n=11, SOC– HF n=12; *triciglerydes* IND–LF n=11, SOC–LF n=10, IND–HF n=11, SOC– HF n=11; *leptin* IND–LF n=12, SOC–LF n=11, IND–HF n=12, SOC– HF n=12. Few samples could not be analysed due to unforeseen circumstances, therefore some groups do not include 12 mice. For insulin and HOMA-IR, data has been log-transformed for statistical analysis, but raw data is graphically represented. Data are expressed as individual data points, means ± standard deviation (SD). P-values are expressed as * (< 0.05), ** (< 0.01) and *** (< 0.001).

Exploratory analysis into the effects of hierarchy and diet (only in socially housed mice)

Social hierarchy was evaluated by means of a tube test performed at PND124-125 in the SOC group only. To exclude that the mouse rank was dependent on diet and

body size, the number of wins (3-5) of dominant animals was used as dependent variable in a general linear model with diet and either body weight, the pair delta body weight (body weight dominant - body weight subordinate), fat mass or lean mass as independent variables. Neither diet nor body weight, delta body weight, fat mass and lean mass predicted the number of wins, indicating that the number of wins was not dependent on the size of dominant animals. Therefore we continued this exploratory analysis by using the social rank of each animal used as a factor (together with diet) for statistical analysis on the parameters investigated. However, in this section, only main effects of hierarchy and eventual interactions (hierarchy x diet) are discussed, as diet has been thoroughly discussed in the previous section.

Body weight

Body weight did not differ at weaning between dominant and subordinate mice ($p = 0.9$). No effects of social hierarchy were found on adolescent body weight gain ($p = 0.2$, figure 8A). However, dominant mice were heavier at PND42 compared to subordinate counterparts ($p = 0.01$). Furthermore, an interaction between social hierarchy and diet was present for adult body weight ($p = 0.01$). Tukey post-hoc test showed that body weight was higher in dominant versus subordinate mice on the HF diet ($p = 0.009$), but this effect was not seen in LF fed mice ($p = 0.99$) (figure 8B).

Bone mineral content and bone mineral density

BMC ($p=0.19$) and BMD ($p = 0.7$) were unaffected by social hierarchy (figure 9).

Body composition analysis

At PND126, body weight in fasted conditions presented a hierarchy x diet interaction ($p = 0.046$), indicating that HF-dominant mice were heavier than HF-subordinate counterparts ($p < 0.001$, figure 10A). Interestingly, these differences were absent between LF-dominant and LF-subordinate mice. Furthermore, dominant mice showed increased absolute fat mass ($p = 0.02$, figure 10B) and absolute lean mass ($p = 0.001$, figure 10D) compared to subordinate animals. Specifically, higher fat mass in dominant mice versus subordinate mice on HF diet was reflected in more retroperitoneal fat ($p = 0.02$), inguinal fat ($p = 0.03$), brown adipose tissue ($p = 0.02$) and muscle and organs fat ($p = 0.03$) (table 4). However, the percentage of fat mass of body weight ($p = 0.06$, figure 10C) and the fat to lean mass ratio ($p = 0.06$, figure 10E) were not significantly affected by hierarchy.

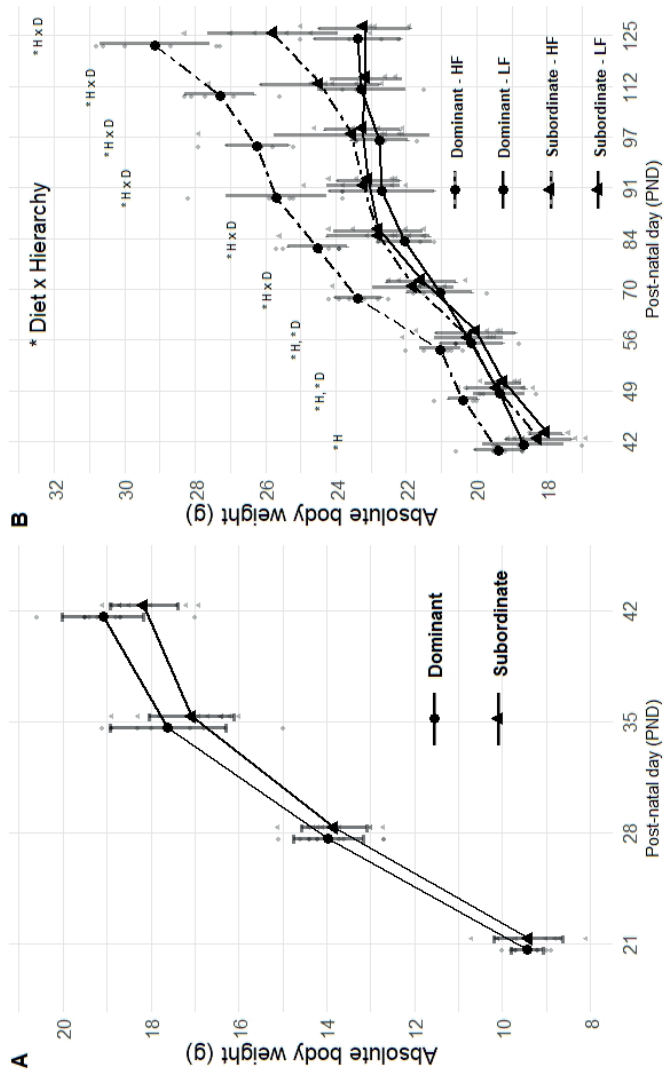

Figure 8. Body weight of dominant and subordinate female mice. (A) Absolute body weight of dominant and subordinate mice in adolescence (PND21-PND42). (B) Absolute body weight of dominant versus subordinate mice fed either a LF and HF diet in adulthood (PND42 - PND126). Data is expressed as individual units, means and standard deviation (SD). H = hierarchy effect, D = diet effect, HxD = hierarchy x diet interaction. P-values are expressed as * (< 0.05), ** (< 0.01) and *** (< 0.001).

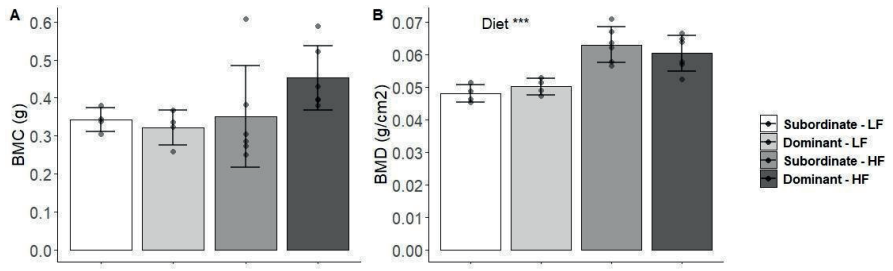

Figure 9. Bone mineral content (A) and bone mineral density (B) assessed by dual energy x-ray absorptiometry (DXA). Data is expressed as individual units, means and standard deviation (SD). (Dominant–LF n=4, Subordinate–LF n=4, Dominant–HF n=6, Subordinate– HF n=6). P-values are expressed as * (< 0.05), ** (< 0.01) and *** (< 0.001).

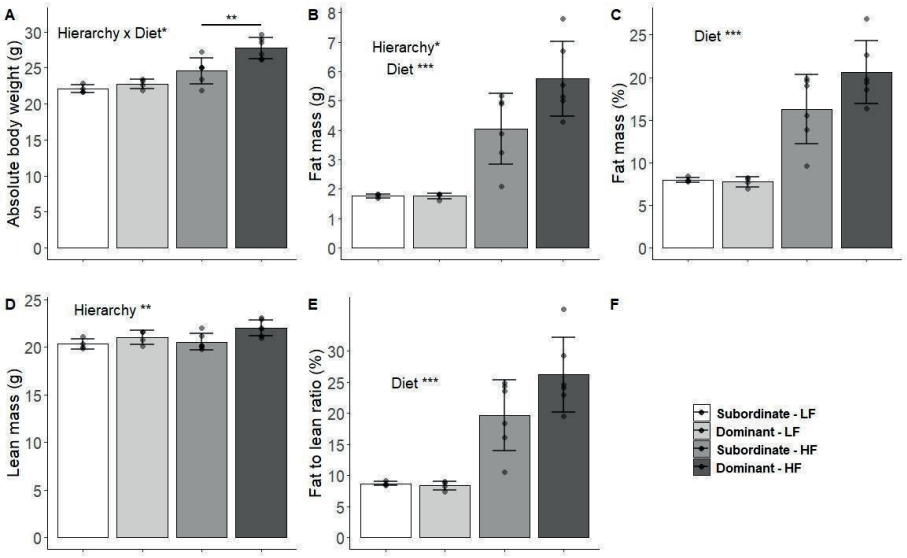

Figure 10. Body composition analysis assessed at PND126 by fat extraction performed with petroleum ether in a soxhlet apparatus. (**A**) Fat mass (g), (**B**) fat mass (%), (**C**) lean mass (g), (**D**) fat to lean mass ratio, (**E**) femur length. Data is expressed as individual datapoints, means ± standard deviation (SD). P-values are expressed as * (< 0.05), ** (< 0.01) and *** (< 0.001).

	Subordinate - LF	Dominant - LF	Subordinate - HF	Dominant - HF	main effect
WAT, perirenal (mg)	49.6 ± 23.5	79.3 ± 44.9	134.8 ± 90	201.1 ± 155.9	diet *
WAT, retroperitoneal (mg)	37 ± 4.3	51 ± 10.5	133.9 ± 65.3	235.1 ± 94.7	hierarchy *, diet ***
WAT, inguinal (mg)	205.2 ± 45.7	248.1 ± 58.8	478.1 ± 179	699.3 ± 204.9	hierarchy *, diet ***
WAT, subcutaneous (mg)	448 ± 49.2	496.9 ± 99.8	1115.6 ± 339.8	1417.4 ± 319.7	diet ***
WAT, muscle & organs fat (mg)	896 ± 44.5	738.7 ± 128.1	1939.4 ± 702.1	2869 ± 603.6	hierarchy x diet (*)
BAT, interscapular (mg)	127.7 ± 9.3	144 ± 36.8	243.1 ± 48	325.2 ± 79.2	hierarchy *, diet ***
Femur length (mm)	15.8 ± 0.4	15.6 ± 0.2	15.7 ± 0.2	15.9 ± 0.2	-

Table 4. Absolute body weight, fat mass, lean mass, fat pads and femur length assessed at PND126 by fat extraction performed with petroleum ether in a soxhlet apparatus. Data is expressed as means ± standard deviation (SD). P-values are expressed as * (< 0.05), ** (< 0.01) and *** (< 0.001).

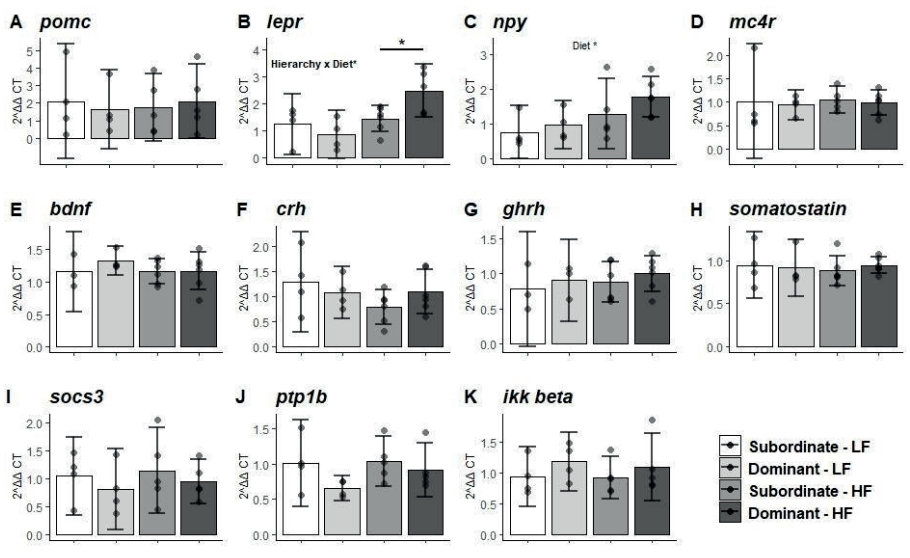

Figure 11. Relative *pomc*, *lepr*, *npy*, *mc4r*, *bdnf*, *crh*, *ghrh*, *somatostatin*, *socs3*, *ptp1b* and *ikk-β* hypothalamic gene expression. Data is expressed as individual units, means ± 95% confidence intervals. (*pomc*, *socs3*, *ptp1b* and *ikk-β*: Subordinate–LF n=4, Dominant–LF n=4, Subordinate– HF n=5, Dominant–HF n=5 – *lepr*: Subordinate–LF n=4, Dominant–LF n=4, Subordinate– HF n=6, Dominant–HF n=5 – *npy and mc4r*: Subordinate–LF n=4, Dominant–LF n=4, Subordinate– HF n=5, Dominant–HF n=6 – *mc4r*: – *bdnf*: Subordinate–LF n=3, Dominant–LF n=4, Subordinate– HF n=6, Dominant–HF n=6 – *crh and somatostatin*: Subordinate–LF n=4, Dominant–LF n=4, Subordinate– HF n=6, Dominant–HF n=6 – *ghrh*: Subordinate–LF n=3, Dominant–LF n=3, Subordinate– HF n=6, Dominant–HF n=6). P-values are expressed as * (< 0.05), ** (< 0.01) and *** (< 0.001).

Hypothalamic gene expression

Hypothalamic gene expression showed a hierarchy x diet interaction on *lepr* expression ($p = 0.015$), indicating that on HF diet dominant mice had higher *lepr* expression than subordinate counterparts ($p = 0.03$), whereas this was not observed in mice on LF diet (figure 11B). In addition, there was a trend of reduced *ptp1b* expression in dominant mice ($p = 0.09$) compared to subordinate mice (figure 11J). The expression of the other genes was unaffected by hierarchy (figure 11).

Plasma levels of glucose, insulin, corticosterone, HOMA-IR, triglycerides and leptin

Plasma levels of insulin, glucose, corticosterone, calculated HOMA-IR and triglycerides were all unaffected by social hierarchy (figure 12). Leptin levels were increased in dominant animals ($p = 0.02$), irrespective of diet type (figure 12 F).

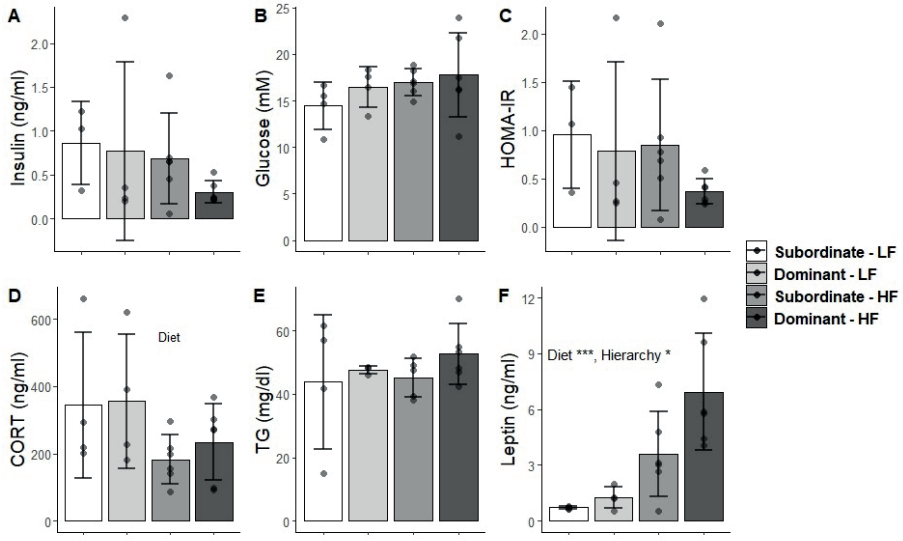

Figure 12. Plasma levels of **(A)** insulin (ng/ml), **(B)** glucose (mM), **(C)** calculated HOMA-IR, **(D)** plasma corticosterone (ng/ml), **(E)** triglycerides (mg/dl) and **(D)** leptin (ng/ml). Group composition is as follows: *insulin* and *HOMA-IR*: Subordinate–LF n=3, Dominant–LF n=4, Subordinate– HF n=6; Dominant–HF n=6; *glucose, CORT* and *leptin:* Subordinate–LF n=4, Dominant–LF n=4, Subordinate–HF n=6, Dominant–HF n=6; *triglycerides*: Subordinate–LF n=4, Dominant–LF n=3, Subordinate– HF n=5, Dominant–HF n=6.. Data has been log-transformed for statistical analysis for insulin,HOMA-IR and CORT, but raw data is graphically represented. Data are expressed as individual data points, means ± standard deviation (SD). P-values are expressed as * (< 0.05), ** (< 0.01) and *** (< 0.001).

Discussion

The present study showed that adult diet, but not postweaning individual housing modulates body weight trajectories and adiposity in female C57BL/6J mice until PND 126. Higher body weight and adiposity was observed in HF fed mice compared to LF fed mice, which could be explained, in part, by higher energy intake in HF mice as frequently shown by others in the past too (Hu et al., 2018; Licholai et al., 2018). Interestingly, a significantly increased food intake in IND housed mice relative to SOC housed mice was observed, which did not pertain to differences in body weight. One explanation relevant to this outcome is that the experiments were carried out at room temperature, which is well below the thermoneutral zone of mice (Reitman, 2018). SOC housed mice frequently huddle, particularly during the resting phase, thereby sharing body heat; a process that is called social thermoregulation (Gilbert et al., 2010). IND housed mice lack this possibility and would therefore emit relatively more heat to the environment than SOC housed, hence the requirement of increased EI to maintain energy balance. While we do not have an account of energy expenditure in the present study to substantiate this point, we did show the presence of such a mechanism in male mice housed at room temperature, in which IND housing of mice caused higher levels of food intake and resting metabolic rate (RMR), relative to SOC housing (chapter 3 of this thesis and Schipper et al., 2020).

A major difference between the female mice in the present study and the male mice discussed in a previous study (chapter 3 of this thesis) was that IND housing caused male mice to decrease body size on the basis of decreased lean mass and femur length, phenomena that were not observed in the females in the present study. In fact, IND housed females in the present study presented a significantly increased, albeit relatively small increase in lean mass, without changes in femur length compared to SOC. Bone mineral density (BMD, on the basis of DXA scanning), on the other hand, was reduced in IND housed female mice in the present study, but only when subjected to a HF diet. In the male mice the effect of IND housing on BMD appeared irrespective of diet. One potential underlying mechanism may be that mechanical loading increases BMD in mice (Kesavan et al, 2005) and IND housing in male mice is thought to reduce mechanical loading as there is no activity due to social conflict (fighting) behavior (Meakin et al, 2013). A lower level of activity in general in IND compared to SOC is probably not playing a role (Shin et al, 2018). Since female mice engage less in aggressive and fighting behaviors, but show larger responses to mechanical loading than male mice (Meakin et al, 2013), it may be speculated that lack of

social (playing) behaviors during adolescence may be sufficient to reduce BMD in IND female mice. It remains unclear however why the effect reached significance in the current study only when females were exposed to HF diet. While these findings are of interest, they are based on DEXA scanning at PND97, and do need confirmation by histological and molecular analysis. In addition, the underlying (brain) mechanisms should be revealed. In this sense, the reduced hypothalamic expression of POMC in IND housed females relative to SOC housed ones which we observed in the present study could be of interest. The melanocortin system, with POMC as one of its regulatory genes (Cone, 2005), has been implicated in bone homeostasis (Idelevich et al., 2018), however with complex interactions between lean mass and adiposity (Butler, 2006). These interactions deserve to be investigated further with emphasis on the role of (social) housing in these mechanisms. The high levels of hypothalamic NPY and leptin receptor expression in the SOC-HF mice in the present study are intriguing as they, based on gene deletion experiments (Wong et al., 2013), would not be expected to be associated with a high level of BMD. They are, however, more in line with exposure to a HF diet leading to increased adiposity (Huang, Han & Storlien, 2003).

Despite the effects of the housing and diet conditions on caloric intake, adiposity, lean mass, and BMD, there were remarkably little effects on associated plasma parameters of fuels and hormones, perhaps due the fact that females are quite resilient to develop cardiometabolic derangements at young age (Jacobs et al., 2019). Despite no changes in the levels of glucose, insulin and triglycerides in response to housing and diet, leptin levels were increased in response to high-fat feeding, suggesting leptin resistance (Knight et al., 2010). However, we did not find changes in hypothalamic genes of interest that could underlie leptin resistance in HF animals, such as *socs3*, *ptp1b* and *ikk beta*. It remains to be seen whether other key genes could support hypothalamic leptin resistance, such as the JAK2-STAT3 pathway (Liu et al., 2021). Another exception was the decreased plasma levels of corticosterone (CORT), which was lower in the IND-LF group compared to SOC-LF, despite no changes in hypothalamic *crh* expression. Although elevated levels of CORT can be seen in response to stress, CORT levels may actually also rise as a result of pleasurable conditions (Koolhaas et al., 2011). In this respect, the absence of a difference between the IND-HF and SOC-HF in plasma CORT levels is of interest. Since HF feeding is known for its blunting effects on corticosterone levels (Auvinen et al., 2012; Hwang et al., 2010), it may be speculated that as female C57BL/6J mice housed in groups seem to present increased levels of corticosterone levels (Arndt et al., 2009), this may have been prevented by the HF diet in SOC housed mice in the present study.

In an exploratory analysis, we investigated the establishment of social dominance relations in SOC housed mice, and its potential relevance (in interaction with diet) for the assessed parameters. At the end of the study, a clear hierarchical dominance/subordination difference between cage mates was revealed by exposing the animals to the tube test. The reason for assessment of social rank in a rather late stage of this experiment is that we did not want to potentially inflate rivalry between the females by subjection to the tube test, as this may have subsequently affected health, weight gain trajectories etc. The disadvantage however is that we do not have an account of dominance status during earlier stages. Several lines of research however indicate that dominance hierarchy is rather stable in small groups of littermates (Bartolomucci et al., 2001) or non-littermates (Vekovishcheva, Sukhotina & Zvartau, 2000), at least in male mice. Analysis of the data according to dominance hierarchy showed that dominant females had higher weight gain relative to subordinate ones irrespective of the number wins in the tube test (i.e., 3,4, or 5), but this effect was only present in HF exposed animals. While the development and dynamics of the dominance hierarchy at earlier stages of life, such as in the litter or post-weaning after PND 21, was not assessed in the current study, retrospective analyses revealed that dominant females had higher body weight than subordinate ones at PND 42, just before they were exposed to the HF diet. Remarkably, the observed effects of hierarchy on parameters of energy balance in female mice were not observed in male mice (see chapter 3 in this thesis), suggesting a female-biased effect of pair housing on HF diet-induced perturbations of energy balance. Dominant females showed an increase in lean mass and fat mass irrespective of diet type, but this did not pertain to difference in femur length or BMD/BMC, suggesting that dominant females are not larger animals per sé, but accrual more fat and lean mass relative to subordinate ones.

Although our study did not allow to dissociate energy intake between dominant and subordinate mice, it may be speculated that dominant mice fed a HF diet ingested more food in relation to metabolism than their subordinate counterparts. An intriguing hypothesis by MacCormack and Muscatell assumes that the leptin pathway is sensitive to social context, thereby affecting metabolic status (MacCormack and Muscatell 2019). Interestingly, dominant female mice showed hyperleptinemia relative to subordinate mice, suggesting that these mice were leptin resistant. However, *lepr* expression in HF dominant female mice waas increased compared to subordinate ones, paradoxically suggesting improved leptin sensitivity. However, other post-receptor mechanisms could still underlie leptin resistance (Gruzdeva et al., 2019). Among the mechanisms that

induce leptin resistance is hypothalamic inflammation, in which intracellular factors such as *socs3* and *ikk-β* mediate leptin resistance (Zhang et al. 2009). Our exploratory analyses could not be conclusive in this respect, as we did not find significant effects of social status on hypothalamic expression of *socs3* and *ikk-β*, however, we did find a trend of reduced *ptp1b* expression in the dominant female mice relative to the subordinate ones. *ptp1b* contributes to leptin resistance by inhibiting intracellular leptin receptor signaling, however, a reduced expression of *ptp1b* may indicate that dominant mice may not have been leptin resistant (White et al, 2009). These results are not conclusive, as our exploratory analysis was performed with a low sample size, and these effects should be studied in the future using different designs with sufficient power. The small sample we used may have prohibited us from having a clear understanding of how hypothalamic gene expression was affected by social hierarchy. In addition, we assessed social hierarchies only at the end of the experiment, and although the available literature suggests that dominance hierarchy is rather stable in small groups in male mice, it remains to be seen whether these hierarchies were present in early life and they were stable throughout an experimental setting as in the present study. However, this exploratory analysis indicates that further analysis of the interplay between dominance hierarchy and diet on the neurobiology of energy balance regulation and beyond is therefore warranted.

In summary, the results of our experiment suggest that differences in energy balance regulation between SOC housed female littermates and IND housed ones are relatively small, certainly in comparison to the impact of feeding a HF diet versus a LF diet. Within-cage differences in energy balance regulation on the basis of dominance hierarchy, however, were found to be quite striking, and were either dependent (i.e., weight gain) or independent (i.e., lean mass and fat mass) of feeding a HF diet.

References

Arndt SS, Laarakker MC, van Lith HA, van der Staay FJ, Gieling E, Salomons AR, van't Klooster J, Ohl F. Individual housing of mice--impact on behaviour and stress responses. *Physiol Behav*. 2009 Jun 22;97(3-4):385-93. doi: 10.1016/j.physbeh.2009.03.008. Epub 2009 Mar 18. PMID: 19303031.

Auvinen HE, Romijn JA, Biermasz NR, Pijl H, Havekes LM, Smit JW, Rensen PC, Pereira AM. The effects of high fat diet on the basal activity of the hypothalamus-pituitary-adrenal axis in mice. *J Endocrinol*. 2012 Aug;214(2):191-7. doi: 10.1530/JOE-12-0056. Epub 2012 May 22. PMID: 22619233.

Barnhart, J. E., and W. J. Pizzi. 1982. "The Monosodium L-Glutamate (MSG) Syndrome in Mice Develops Independently of Housing Condition." *Neurobehavioral Toxicology and Teratology* 4(5): 549–56. https://europepmc.org/article/med/7177307 (March 18, 2021).

Bartolomucci A, Palanza P, Gaspani L, Limiroli E, Panerai AE, Ceresini G, Poli MD, Parmigiani S. Social status in mice: behavioral, endocrine and immune changes are context dependent. *Physiol Behav*. 2001 Jun;73(3):401-10. doi: 10.1016/s0031-9384(01)00453-x. PMID: 11438368.

Berry, Alessandra et al. 2012. "Social Deprivation Stress Is a Triggering Factor for the Emergence of Anxiety- and Depression-like Behaviours and Leads to Reduced Brain BDNF Levels in C57BL/6J Mice." *Psychoneuroendocrinology* 37(6): 762–72. http://dx.doi.org/10.1016/j.psyneuen.2011.09.007.

Blanchard, D. Caroline et al. 1995. "Visible Burrow System as a Model of Chronic Social Stress: Behavioral and Neuroendocrine Correlates." *Psychoneuroendocrinology* 20(2): 117–34.

Butler AA. The melanocortin system and energy balance. *Peptides*. 2006 Feb;27(2):281-90. doi: 10.1016/j.peptides.2005.02.029. Epub 2006 Jan 23. PMID: 16434123; PMCID: PMC2735083.

Chvédoff, M. et al. 1980. "Effects of Housing Conditions on Food Intake, Body Weight and Spontaneous Lesions in Mice. A Review of the Literature and Results of an 18-Month Study." *Food and Cosmetics Toxicology* 18(5): 517–22.

Collins S, Martin TL, Surwit RS, Robidoux J. Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. *Physiol Behav*. 2004 Apr;81(2):243-8. doi: 10.1016/j.physbeh.2004.02.006. PMID: 15159170.

Collura, Lynn A., Jackie B. Hoffman, and Mark E. Wilson. 2009. "Administration of Human Leptin Differentially Affects Parameters of Cortisol Secretion in Socially Housed Female Rhesus Monkeys." *Endocrine* 36(3): 530–37.

Cone RD. Anatomy and regulation of the central melanocortin system. *Nat Neurosci*. 2005 May;8(5):571-8. doi: 10.1038/nn1455. PMID: 15856065.

Dadomo, Harold et al. 2011. "Vulnerability to Chronic Subordination Stress-Induced Depression-like Disorders in Adult 129SvEv Male Mice." *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 35(6): 1461–71.

Epel, Elissa, Rachel Lapidus, Bruce McEwen, and Kelly Brownell. 2001. "Stress May Add Bite to Appetite in Women: A Laboratory Study of Stress-Induced Cortisol and Eating Behavior." *Psychoneuroendocrinology* 26(1): 37–49.

Gifford, Robert M., and Rebecca M. Reynolds. 2017. "Sex Differences in Early-Life Programming of the Hypothalamic–Pituitary–Adrenal Axis in Humans." *Early Human Development* 114: 7–10.

Gilbert C, McCafferty D, Le Maho Y, Martrette JM, Giroud S, Blanc S, Ancel A. One for all and all for one: the energetic benefits of huddling in endotherms. *Biol Rev Camb Philos Soc*. 2010 Aug;85(3):545-69. doi: 10.1111/j.1469-185X.2009.00115.x. Epub 2009 Dec 21. PMID: 20039866.

Gruzdeva O, Borodkina D, Uchasova E, Dyleva Y, Barbarash O. Leptin resistance: underlying mechanisms and diagnosis. *Diabetes Metab Syndr Obes*. 2019 Jan 25;12:191-198. doi: 10.2147/DMSO.S182406. PMID: 30774404; PMCID: PMC6354688.

Guo, Ming et al. 2004. "Sex Difference in Psychological Behavior Changes Induced by Long-Term Social Isolation in Mice." *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 28(1): 115–21.

Hu S, Wang L, Yang D, Li L, Togo J, Wu Y, Liu Q, Li B, Li M, Wang G, Zhang X, Niu C, Li J, Xu Y, Couper E, Whittington-Davies A, Mazidi M, Luo L, Wang S, Douglas A, Speakman JR. Dietary Fat, but Not Protein or Carbohydrate, Regulates Energy Intake and Causes Adiposity in Mice. *Cell Metab*. 2018 Sep 4;28(3):415-431.e4. doi: 10.1016/j.cmet.2018.06.010. Epub 2018 Jul 12. PMID: 30017356.

Huang XF, Han M, Storlien LH. The level of NPY receptor mRNA expression in diet-induced obese and resistant mice. *Brain Res Mol Brain Res*. 2003 Jul 4;115(1):21-8. doi: 10.1016/s0169-328x(03)00174-8. PMID: 12824051.

Hwang LL, Wang CH, Li TL, Chang SD, Lin LC, Chen CP, Chen CT, Liang KC, Ho IK, Yang WS, Chiou LC. Sex differences in high-fat diet-induced obesity, metabolic alterations and learning, and synaptic plasticity deficits in mice. *Obesity (Silver Spring)*. 2010 Mar;18(3):463-9. doi: 10.1038/oby.2009.273. Epub 2009 Sep 3. PMID: 19730425.

Idelevich A, Sato K, Nagano K, Rowe G, Gori F, Baron R. Neuronal hypothalamic regulation of body metabolism and bone density is galanin dependent. *J Clin Invest*. 2018 Jun 1;128(6):2626-2641. doi: 10.1172/JCI99350. Epub 2018 May 14. PMID: 29596063; PMCID: PMC5983337.

Isasi, Carmen R et al. 2015. "Psychosocial Stress Is Associated with Obesity and Diet Quality in Hispanic/Latino Adults." *Ann Epidemiol* 25(2): 84–89.

Jacobs SAH, Gart E, Vreeken D, Franx BAA, Wekking L, Verweij VGM, Worms N, Schoemaker MH, Gross G, Morrison MC, Kleemann R, Arnoldussen IAC, Kiliaan AJ. Sex-Specific Differences in Fat Storage, Development of Non-Alcoholic Fatty Liver Disease and Brain Structure in Juvenile HFD-Induced Obese Ldlr-/-.Leiden Mice. *Nutrients*. 2019 Aug 10;11(8):1861. doi: 10.3390/nu11081861. PMID: 31405127; PMCID: PMC6723313.

Jahng, Jeong Won, Sang Bae Yoo, Vitaly Ryu, and Jong Ho Lee. 2012. "Hyperphagia and Depression-like Behavior by Adolescence Social Isolation in Female Rats." *International Journal of Developmental Neuroscience* 30(1): 47–53. http://dx.doi.org/10.1016/j.ijdevneu.2011.10.001.

Jarrell, Holly et al. 2008. 93 Physiol Behav *Polymorphisms in the Serotonin Reuptake Transporter Gene Modify the Consequences of Social Status on Metabolic Health in Female Rhesus Monkeys*. http://www.elsevier.com/copyright (March 16, 2021).

Kappel, Sarah, Penny Hawkins, and Michael T. Mendl. 2017. "To Group or Not to Group? Good Practice for Housing Male Laboratory Mice." *Animals* 7(12): 1–25.

Kesavan C, Mohan S, Oberholtzer S, Wergedal JE, Baylink DJ. Mechanical loading-induced gene expression and BMD changes are different in two inbred mouse strains. *J Appl Physiol* (1985). 2005 Nov;99(5):1951-7. doi: 10.1152/japplphysiol.00401.2005. Epub 2005 Jul 14. PMID: 16024522.

Knight ZA, Hannan KS, Greenberg ML, Friedman JM. Hyperleptinemia is required for the development of leptin resistance. *PLoS One*. 2010 Jun 29;5(6):e11376. doi: 10.1371/journal.pone.0011376. PMID: 20613882; PMCID: PMC2894068.

Koolhaas JM, Bartolomucci A, Buwalda B, de Boer SF, Flügge G, Korte SM, Meerlo P, Murison R, Olivier B, Palanza P, Richter-Levin G, Sgoifo A, Steimer T, Stiedl O, van Dijk G, Wöhr M, Fuchs E. Stress revisited: a critical evaluation of the stress concept. *Neurosci Biobehav Rev*. 2011 Apr;35(5):1291-301. doi: 10.1016/j.neubiorev.2011.02.003. Epub 2011 Feb 21. PMID: 21316391.

Licholai JA, Nguyen KP, Fobbs WC, Schuster CJ, Ali MA, Kravitz AV. Why Do Mice Overeat High-Fat Diets? How High-Fat Diet Alters the Regulation of Daily Caloric Intake in Mice. *Obesity (Silver Spring)*. 2018 Jun;26(6):1026-1033. doi: 10.1002/oby.22195. Epub 2018 Apr 29. PMID: 29707908; PMCID: PMC5970071.

Liu H, Du T, Li C, Yang G. STAT3 phosphorylation in central leptin resistance. *Nutr Metab* (Lond). 2021 Apr 13;18(1):39. doi: 10.1186/s12986-021-00569-w. PMID: 33849593; PMCID: PMC8045279.

Lopez, Marcelo F., and Kathy Laber. 2015. "Impact of Social Isolation and Enriched Environment during Adolescence on Voluntary Ethanol Intake and Anxiety in C57BL/6J Mice." *Physiology and Behavior* 148: 151–56. http://dx.doi.org/10.1016/j.physbeh.2014.11.012.

MacCormack, Jennifer K., and Keely A. Muscatell. 2019. "The Metabolic Mind: A Role for Leptin and Ghrelin in Affect and Social Cognition." *Social and Personality Psychology Compass* 13(9): 1–18.

McCormick, Cheryl M., and Iva Z. Mathews. 2007. "HPA Function in Adolescence: Role of Sex Hormones in Its Regulation and the Enduring Consequences of Exposure to Stressors." *Pharmacology Biochemistry and Behavior* 86(2): 220–33.

Meakin LB, Sugiyama T, Galea GL, Browne WJ, Lanyon LE, Price JS. Male mice housed in groups engage in frequent fighting and show a lower response to additional bone loading than females or individually housed males that do not fight. *Bone*. 2013 May;54(1):113-7. doi: 10.1016/j.bone.2013.01.029. Epub 2013 Jan 26. PMID: 23356987; PMCID: PMC3607215.

Michopoulos, Vasiliki, Melinda Higgins, Donna Toufexis, and Mark E. Wilson. 2012. "Social Subordination Produces Distinct Stress-Related Phenotypes in Female Rhesus Monkeys." *Psychoneuroendocrinology* 37(7): 1071–85.

Michopoulos, Vasiliki, and Mark E. Wilson. 2011. "Body Weight Decreases Induced by Estradiol in Female Rhesus Monkeys Are Dependent upon Social Status." *Physiology and Behavior* 102(3–4): 382–88. http://dx.doi.org/10.1016/j.physbeh.2010.11.031.

Moles, A. et al. 2006. "Psychosocial Stress Affects Energy Balance in Mice: Modulation by Social Status." *Psychoneuroendocrinology* 31(5): 623–33.

Núñez NP, Carpenter CL, Perkins SN, Berrigan D, Jaque SV, Ingles SA, Bernstein L, Forman MR, Barrett JC, Hursting SD. Extreme obesity reduces bone mineral density: complementary evidence from mice and women. *Obesity (Silver Spring)*. 2007 Aug;15(8):1980-7. doi: 10.1038/oby.2007.236. PMID: 17712115.

R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: http://www.R-project.org/.

Reijne, Aaffien C. et al. 2016. "Whole-Body Vibration Partially Reverses Aging-Induced Increases in Visceral Adiposity and Hepatic Lipid Storage in Mice." *PLoS ONE* 11(2): 1–18.

Reitman ML. Of mice and men - environmental temperature, body temperature, and treatment of obesity. *FEBS* Lett. 2018 Jun;592(12):2098-2107. doi: 10.1002/1873-3468.13070. Epub 2018 May 10. PMID: 29697140.

Schipper, L., L. Harvey, E. M. van der Beek, and G. van Dijk. 2018. "Home Alone: A Systematic Review and Meta-Analysis on the Effects of Individual Housing on Body Weight, Food Intake and Visceral Fat Mass in Rodents." *Obesity Reviews*. http://www.ncbi.nlm.nih.gov/pubmed/29334694%0Ahttp://doi.wiley.com/10.1111/obr.12663.

Schipper, Lidewij et al. 2020. "Individual Housing of Male C57BL/6J Mice after Weaning Impairs Growth and Predisposes for Obesity." *PLoS ONE* 15(5): 1–17. http://dx.doi.org/10.1371/journal.pone.0225488.

Shin J, Woo J, Cho Y, Choi YH, Shin NN, Kim Y. Four-week individual caging of male ICR mice alters body composition without change in body mass. *Sci Rep.* 2018 Jan 22;8(1):1331. doi: 10.1038/s41598-018-19808-x. PMID: 29358648; PMCID: PMC5778080.

Smith, J. T., and B. J. Waddell. 2003. "Developmental Changes in Plasma Leptin and Hypothalamic Leptic Receptor Expression in the Rat: Peripubertal Changes and the Emergence of Sex Differences." *Journal of Endocrinology* 176(3): 313–19.

Solomon, Matia B., and James P. Herman. 2009. "Sex Differences in Psychopathology: Of Gonads, Adrenals and Mental Illness." *Physiology and Behavior* 97(2): 250–58. http://dx.doi.org/10.1016/j.physbeh.2009.02.033.

Tamashiro, Kellie L.K. et al. 2004. "Metabolic and Endocrine Consequences of Social Stress in a Visible Burrow System." *Physiology and Behavior* 80(5): 683–93.

Tschöp, Matthias H. et al. 2012. "A Guide to Analysis of Mouse Energy Metabolism." Nature Methods 9(1): 57–63.

Tortoriello DV, McMinn JE, Chua SC. Increased expression of hypothalamic leptin receptor and adiponectin accompany resistance to dietary-induced obesity and infertility in female C57BL/6J mice. *Int J Obes* (Lond). 2007 Mar;31(3):395-402. doi: 10.1038/sj.ijo.0803392. Epub 2006 Jul 25. PMID: 16865100.

van Dijk TH, Laskewitz AJ, Grefhorst A, Boer TS, Bloks VW, Kuipers F, Groen AK, Reijngoud DJ. A novel approach to monitor glucose metabolism using stable isotopically labelled glucose in longitudinal studies in mice. *Lab Anim*. 2013 Apr;47(2):79-88. doi: 10.1177/0023677212473714. Epub 2013 Mar 14. PMID: 23492513.

Van Loo PL, Van Zutphen LF, Baumans V. Male management: Coping with aggression problems in male laboratory mice. *Lab Anim*. 2003 Oct;37(4):300-13. doi: 10.1258/002367703322389870. PMID: 14599305.

Vekovishcheva OY, Sukhotina IA, Zvartau EE. Co-housing in a stable hierarchical group is not aversive for dominant and subordinate individuals. *Neurosci Behav Physiol*. 2000 Mar-Apr;30(2):195-200. doi: 10.1007/BF02463158. PMID: 10872730.

Võikar V, Polus A, Vasar E, Rauvala H. Long-term individual housing in C57BL/6J and DBA/2 mice: assessment of behavioral consequences. Genes Brain Behav. 2005 Jun;4(4):240-52. doi: 10.1111/j.1601-183X.2004.00106.x. PMID: 15924556.

Wang F, Kessels HW, Hu H. The mouse that roared: neural mechanisms of social hierarchy. *Trends Neurosci*. 2014 Nov;37(11):674-82. doi: 10.1016/j.tins.2014.07.005. Epub 2014 Aug 24. PMID: 25160682.

Weltman, A. S., A. M. Sachler, and S. B. Sparber. 1966. "Endocrine, Metabolic and Behavioral Aspects of Isolation Stress on Female Albino Mice." *Aerospace medicine* 37(8): 804–10. https://europepmc.org/article/med/5969440 (March 18, 2021).

White CL, Whittington A, Barnes MJ, Wang Z, Bray GA, Morrison CD. HF diets increase hypothalamic PTP1B and induce leptin resistance through both leptin-dependent and -independent mechanisms. Am J Physiol Endocrinol Metab. 2009 Feb;296(2):E291-9. doi: 10.1152/ajpendo.90513.2008. Epub 2008 Nov 18. PMID: 19017730; PMCID: PMC2645015.

Wiberg, G. S., J. M. Airth, and H. C. Grice. 1966. "Methodology in Long-Term Toxicity Tests: A Comparison of Individual versus Community Housing." *Food and Cosmetics Toxicology* 4(C): 47–55.

Williamson CM, Lee W, DeCasien AR, Lanham A, Romeo RD, Curley JP. Social hierarchy position in female mice is associated with plasma corticosterone levels and hypothalamic gene expression. Sci Rep. 2019 May 13;9(1):7324. doi: 10.1038/s41598-019-43747-w. PMID: 31086272; PMCID: PMC6513839.

Wilson, Mark E. et al. 2008. "Quantifying Food Intake in Socially Housed Monkeys: Social Status Effects on Caloric Consumption." *Physiology and Behavior* 94(4): 586–94.

Wong IP, Nguyen AD, Khor EC, Enriquez RF, Eisman JA, Sainsbury A, Herzog H, Baldock PA. Neuropeptide Y is a critical modulator of leptin's regulation of cortical bone. *J Bone Miner Res*. 2013 Apr;28(4):886-98. doi: 10.1002/jbmr.1786. PMID: 23044938.

Zhang, Xiaoqing et al. 2009. "Hypothalamic IKKb/NF-KB and ER Stress Link Overnutrition." *October* 135(1): 61–73.

Zidar J, Weber EM, Ewaldsson B, Tjäder S, Lilja J, Mount J, Svensson C, Svensk E, Udén E, Törnqvist AE. Group and Single Housing of Male Mice: Collected Experiences from Research Facilities in Sweden. *Animals* (Basel). 2019 Nov 21;9(12):1010. doi: 10.3390/ani9121010. PMID: 31766349; PMCID: PMC6940771.