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

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Chapter 3

Post-weaning individual housing of C57BL/6J male mice affects growth trajectories and energy balance, irrespective of environmental temperature

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Abstract

Rodent studies that model metabolic dysfunctions differ widely in study design, including (social) housing conditions. Social thermoregulation is a strategy applied by socially housed mice kept at standard room temperature (21°C, i.e. below thermoneutrality) to minimize energy use for body temperature regulation. Individual housing at 21°C has been hypothesized to affect energy balance and metabolic health due to lack of social thermoregulation, whereas these effects could be potentially eliminated at thermoneutrality. The current study investigated the consequences of post-weaning individual (IND) versus social (SOC; n=2 per cage) housing at 21°C and at thermoneutrality (28°C) in male C57BL/6J mice, under normal (LF) and high fat diet (HF) conditions. Readouts of body growth including bone mineral content and bone mineral density were evaluated with dual energy absorptiometry (DXA) at postnatal day 97 (PND97) and femur length was assessed at PND126. Body weight was monitored throughout the experiment and energy expenditure and energy intake were determined using indirect calorimetry at PND 113-119. Body composition was determined at PND126 with fat extraction using a soxhlet apparatus. Plasma hormones and hypothalamic gene expression analyses were performed in samples obtained at PND126. IND at 21°C profoundly reduced growth rate and lean mass whereas adiposity was increased. IND increased energy expenditure at an environmental temperature of 21°C suggesting increased thermogenesis. Environmental temperature of 28°C prevented the increased energy expenditure due to IND, but it did not prevent reduced growth and adiposity. This suggests that the metabolic consequences of IND housing are not (only) explained by lack of social thermoregulation. Furthermore, increased environmental temperature to 28°C led to decreased energy intake and expenditure, whereas a HF diet induced an obesity phenotype. These data show that (social) housing and diet could influence experimental outcomes in mouse models of human metabolic dysfunctions.

Introduction

Mice are widely used to model human health and disease development (Speakman and al., 2008). Recommendations for housing and husbandry of laboratory rodents state that they should be group-housed in order to meet the social needs of these species (Council 2011). However, researchers frequently choose to house their mice individually (IND) for a number of reasons. For instance, IND housing makes the assessment of physiological, metabolic and behavioural parameters easier compared to assessing these in SOC housing, as it can be challenging in the latter condition to differentiate between individuals, especially when measuring individual parameters such as food intake and energy expenditure (ref, Tschöp et al. 2012). One solution is to treat each cage with more than one animal as an entity (Schipper et al., 2020), however this leads to ignoring within-cage variance with respect to behaviour (Berry et al. 2012) or energy balance parameters (Moles et al. 2006; Nagy et al. 2002).

While IND housing has a number of (experimental) benefits, it may however impede affective functioning and have consequences for thermoregulation (Schipper et al., 2018). IND mice do not have the ability to huddle and share body heat, a strategy applied by rodents to limit heat loss (Ebensperger, 2001), which might have implications for energy balance regulation per se. Recently, we showed that IND housed mice compared to SOC housing at room temperature (21°C; RT) caused mice to increase (adult) adiposity, which was accompanied by increased energy expenditure and energy intake (Schipper et al. 2020). We hypothesized that the increased adiposity was an adaptation to deal with the increased heat loss to the environment. Increased heat loss to the environment can be compensated by active heat production, and we indeed found that IND mice housed at RT showed increased expression of uncoupling protein-1 (UCP-1) in the brown adipose tissue (BAT) at adolescence and in the inguinal adipose (iWAT) tissue at adulthood (Schipper et al. 2020). This reinforces the idea that social thermoregulation is an adaptive strategy applied by mice to minimize heat loss and thereby lowering energy expenditure in mice at RT (Gilbert et al. 2010).

Because RT is much closer to thermoneutrality in humans (Pallubinsky, Schellen & van Marken Lichtenbelt, 2019) than in (individually housed) mice, the translational value of mice housed at RT to model human health and disease may be questionable (Karp 2012; Ganeshan and Chawla 2017; Hankenson et al. 2018), and is therefore subject to debate (Keijer, Li, and Speakman 2019; Speakman and Keijer 2013). To shed light on this issue, the aim of the present study was to investigate

the consequences of the social housing condition and ambient temperature on a number of energy balance-related health parameters in mice. To achieve this, we compared male mice housed either IND (n=1 per cage) or SOC (n=2 per cage) from weaning till PND 126, either at standard room temperature (21°C) or at a temperature closer to rodent thermoneutrality (28°C). To induce a health-impeding stimulus, half of the above-mentioned cohorts of mice were exposed to either a cafeteria diet high in fat and sucrose (HF diet) from postnatal day (PND) 42 onwards, while the other half remained on the low fat (LF) maintenance diet (which is considered healthy). We subsequently assessed body weight changes, body composition, bone homeostasis, energy intake, energy expenditure, hypothalamic gene expression and plasma hormones and fuels in the mice. We hypothesized that by maintaining IND housed mice at 28°C, the need for social thermoregulation would be reduced, thereby minimizing some of the metabolic consequences of IND housing typically observed at standard RT (like increased adiposity).

Abbreviations

Standard room temperature (RT), Postnatal day (PND), energy expenditure (EE), resting metabolic rate (RMR), energy intake (EI), individual housing (IND), social housing (SOC), low-fat diet (LF), high-fat and high sugar diet (HF), indirect calorimetry (InCA), body weight (BW), SOP dual energy x-ray absorptiometry (DXA), fat mass (FM), body lean mass (LM), percentage of total fat mass respective to BW (%FM), percentage of body lean mass respective to BW (%LM), bone mineral content (BMC), bone mineral density (BMD), fecal testosterone metabolites (FCM).

Material and methods

Ethical statement

This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals established by the Animal Ethics Committee of the University of Groningen, and all animal procedures related to the purpose of the research were approved under the Ethical license of the national competent authority (CCD, Centrale Commissie Dierproeven), securing full compliance the European Directive 2010/63/EU for the use of animals for scientific purposes. C57BL/6J mice were chosen as research subjects as they are the most common inbred strain used in biomedical research. In particular, this strain is prone to obesity and related cardiometabolic alterations (Collins et al., 2004) and were also used in our previous study on the effects of postweaning individual housing versus social housing on sustainable metabolic health (Schipper et al. 2020).

Animals

All mice were housed in polycarbonate type II cages with bedding (Aspen wood shaving) and a shelter (red-transparent plastic house; Techniplast, Varese, Italy). They were in-house bred offspring from primiparous C57BL/6J dams (n=80) and males (n=40) (Charles River Laboratories Sulzfeld, Germany), which were fed a standard rodent diet (Low fat - LF; Altromin® 1410 – 10 mm pellets) and water ad libitum. Before mating, they were habituated in our animal facilities for at least two weeks in climate rooms (21 ± 1°C; 50 ± 5% humidity; 12/12 light/dark cycle with lights on at 09:00), separate rooms were used per sex. Males were housed individually and dams in pairs. Mating took place by hosting a male and a dam together for 72 hours in the climate room assigned to the males. After mating, dams were returned to their room and pair-housed with the same previous cage-mate for two weeks. Pregnancy was determined by an increased body weight of >20% and when pregnant, dams were single housed for the last week of gestation. Body weight (BW) was measured weekly during gestation. At postnatal day 2 (PND2), litters were randomized and culled to 6 pups (4 males and 2 females or 3 males and 3 females) per dam to reduce potential variability based on litter size. Litter weight as a whole was measured weekly during lactation. In total, 96 male C57BL/6J experimental mice were obtained from eight different breeding batches that were performed once a week. On average, 12 mice were obtained for each batch. Batch is here defined as a round of breeding where 10 dams and 10 males were used and offspring were used for the experiment. Dams were used only once and male breeders were used twice. Mice were tail-handled from the base of the tail during the course of this experiment. An outline of the experiment is presented in figure 1.

Housing conditions

Offspring was weaned at postnatal day (PND) 21, and males were randomly housed in one of the following conditions: a) individually housed (IND) at 21°C (n=24), b) socially-housed with a male littermate (SOC, 2 animals per cage) at 21°C (n=24), c) IND at 28°C (n=24), d) SOC at 28°C (n=24). Weaned male mice were housed in a room where only male mice were present. Each group included a total of 24 animals. Climate rooms were used to keep temperature and humidity constant (21 ± 1°C or 28 ± 1°C; 50 ± 5% humidity) and animals were kept in a 12/12 light/dark cycle with lights on at 09:00. Animal procedures and handling took place during the light inactive phase. Cage cleaning took place every two weeks, where olfactory cues (i.e., a handful of wood shavings) were transferred from the old to the new cages in order to maintain familiar odors in the new cage. A red-plastic shelter and the lid containing the food hopper were not replaced/cleaned. Water was provided freshly once a week.

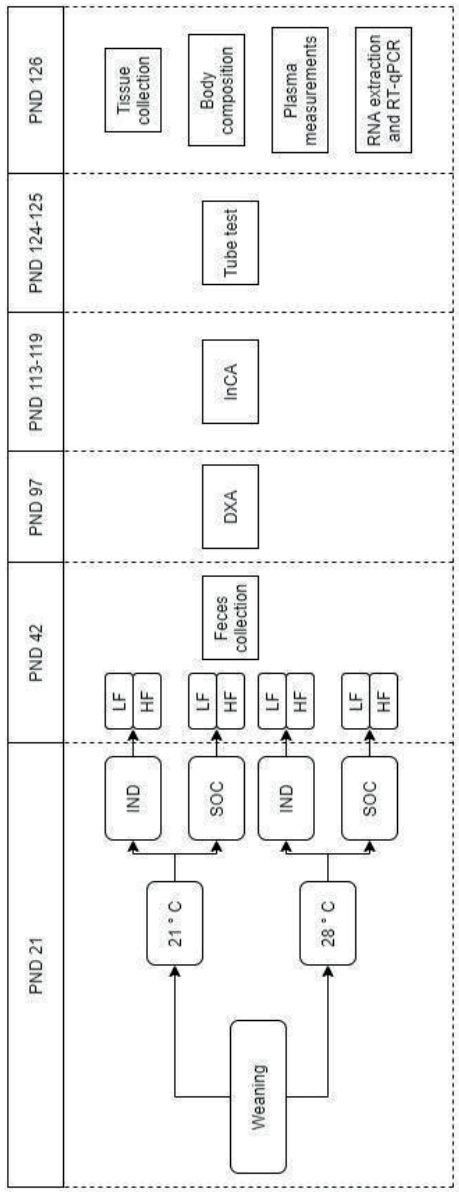

Figure 1. Experimental outline of the experiment. Postnatal dat (PND), individual housing (IND), social housing (SOC), low fat diet (LF), high fat diet (HF), dual energy x-ray absorptiometry (DXA) and indirect calorimetry (InCA).

Diets

After weaning, all groups were kept on the LF. From PND 42 onwards, groups were either exposed to a high fat and high sugar diet (HF) or continued on the LF until the end of the study. The allocation to the groups was random. This gave rise to 8 groups of n=12 each (e.g. 21–IND–LF, 21–SOC–LF, 21–IND–HF, 21–SOC– HF, 28–IND–LF, 28–SOC–LF, 28–IND–HF, 28–SOC–HF). Researchers were aware of the group allocation, as housing, diet and temperature could not be blinded. Diets were provided ad libitum unless specified otherwise. The HF diet was made in-house by using grinded Altromin® 1410 (46.5% of total weight) and by adding lard (14.5%), soy oil (4.7%), sucrose (17.4%), arabic gum (2.3%), casein (10.5%), mineral mix (2.3%) and vitamin mix (1.7%). Minerals and vitamins were added to avoid any deficiency (Weiskirchen et al. 2020). Both the diets were provided as pellets. To avoid fat oxidation, the HF was kept in a freezer (-18°C) and thawed for 2 hours prior to the exposure to the mice. Due to rancidification of the HF diet, this diet was entirely replaced once a week, whereas the LF was refilled once a week. 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 once every two weeks from PND56 until the end of the experiment (PND125). Adolescence body weight gain has been calculated by the following formula: BW PND42 – BW PND21. Adult body weight gain was calculated by the following formula: BW PND125 – BW PND42. From PND21 till PND 42 a total of 24 mice were included in each group (21-SOC; 21-IND; 28-SOC; 28-IND) and from PND42 till PND125a total of 12 mice were included in each group (21–IND–LF, 21–SOC–LF, 21–IND–HF, 21–SOC– HF, 28–IND–LF, 28–SOC–LF, 28–IND–HF, 28–SOC–HF). A few data points are missing in the body weight measurements (two at PND112 and thirteen at PND125) due to unforeseen technical issues.

Feces collection and testosterone metabolite analysis

At PND42, mice were shortly introduced in a new cage until two fecal pellets were present in the wood shaving. This phase lasted on average 3 minutes. In the case no fecal pellets were detected after 5 minutes, the mice were returned to their home-cage and no pellets were collected. As a result, the total sample size consisted of 20 mice per group. Samples were dried for three days at 37°C and then grounded to powder and weighed. Subsequently, the samples were submerged in 1ml methanol, vortexed and let dissolve for 36 hours. The samples were then centrifuged and 0.75ml of the supernatant was collected and evaporated to dryness. The crystalized materials were dissolved in 1ml of assay buffer (pH 7,4) provided with the commercial testosterone kit (DetectX®, Testosterone EIA, Arbor Assays, Ann Arbor, USA). Samples were then treated and analyzed following the manufacturer protocol. Fecal testosterone metabolites (FCM) are expressed as picograms per milliliter (pg/ml) present in the 1ml buffer, obtained through the following formula: *conc(pg/ml)/[0.75 x weight (mg)]*.

Dual energy x-ray absorptiometry (DXA)

At PND 97, bone homeostasis was analyzed by dual energy x-ray absorptiometry (DXA). A pDEXA apparatus from Norland Stratec was used. 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 while 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, the mouse was removed from the apparatus and let recover alone in a cage provided with a heating mat for 10-15 minutes. After complete awakening, the mouse was reintroduced in its home-cage (with or without cage-mate). Bone mineral content (BMC) is reported in grams and bone mineral density (BMD) is reported in grams/cm^2. Due to technical issues, some data points were excluded (21–IND–LF n=9; 21–SOC–LF n=10; 21–IND–HF n=12; 21–SOC– HF n=12; 28–IND–LF n=10; 28–SOC–LF n=8; 28–IND–HF n=12; 28–SOC–HF n=12).

Indirect calorimetry (InCA)

Between PND 113 and 119, mice were moved to a different climate room and each placed within their home cage inside an air-tight box. These boxes were thoroughly cleaned after every use with 70% ethanol and let evaporate for at least 5 minutes. The boxes were connected to a paramagnetic O_2 analyzer (Sevomex Xentra 4100, Crowborough, UK) and by a CO_2 infrared gas analyzer (Servomex 1440). Due to the presence of only one climate room provided with an InCA apparatus, this test had to be performed once at 21°C and once at 28°C each week. This means that of the 8 batches with an average of 12 mice per week, the test was performed once at each temperature, with the order in which the temperature was tested switched every week. This implies that the first temperature was tested between PND 113 and PND 116, and the second between PND 116 and PND 119. This indirect calorimetry system recorded the differential in O_2 and CO_2 between dried reference air and dried air from metabolic chambers. O_2 and CO_2 were calibrated with knowing concentrations of these gases. Flow rate of inlet air was measured with a mass-flow controller (Type 5850 Brooks mass flow controller, Rijswijk, NL) and set at 30 l h^{-1}. The RQ was defined as rCO_2/rO_2 according to Weir (Weir 1949). Energy expenditure (EE) (KJ/time unit) was calculated using the formula: EE = {[(RQ – 0,70) / 0,30] x 473} + {[(1,0 – RQ) / 0,30] x 439} x VO_2. For calculation of resting metabolic rate (RMR) and daily EE, the first day was excluded from the calculations to minimize bias due to the habituation to the novel environment and only the last 48 hours were taken into account. RMR was calculated as the average of the lowest three EE found for each day (that were calculated over 10 minutes) and then averaged over 24 hours. Total EE was calculated by summing the total energy expenditure of day 2 and 3 and then averaged to 24 hours. Energy intake (EI) in the indirect calorimeter system was measured by weighing the food in the food hopper prior and at the end of the test. EE, EI and RMR were calculated either per gram of body weight (average of the BW at the beginning and end of the test) or per gram of lean mass (LM; assessed at PND126). Socially housed mice were not separated in the indirect calorimeter, thus RMR and total EE were calculated per pair and then averaged to one value (n=6), by dividing EE, EI and RMR by two. EE, EI and RMR of socially housed mice were calculated by taking into account the average BW or LM of the pairs. In total, 4 data points could not be used for statistical analysis for EE and RMR due to a software error and 3 for EI due to unforeseen technical issues.

Tube test

Most stable dominance hierarchies develop when mice are housed in small groups (Poole & Morgan, 1973; van Loo et al., 2001). Because dominance in mice relies on prior experience, we decided to perform the tube test later in life between PND124-125 (only for the SOC group), as not to inflate the dominance hierarchy early life (van den Berg, Lamballais & Kushner, 2015). Corresponding to the differences in body size between HF-fed and LF-fed mice, two different tubes were used: one with an inside diameter of 33mm and with an inside diameter of 30mm respectively. 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. 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 cagemate. Win is here defined as the mouse that is able to push the other mouse out of the tube. The mouse that won more trials was considered the dominant, while the losing mouse was considered the subordinate. This test was validated in our own facilities. More information on the tube test can be found elsewhere (Fan et al., 2019).

Tissue collection and body composition analysis

Before tissue collection on PND 126 mice were weighed at the previous day (at 17:00 hrs) and were provided with half the amount of their normal nocturnal food intake, by taking into account the food eaten for three consecutive evenings/ nights between PND91-97. This was done to provide a semi-fasted state at sacrifice. To reduce differences in food eaten between cage-mates, we evenly distributed small pellets of food over the cage and the body weight of the pairs were monitored. Delta body weight analysis did not show differences in body weight lost during the last night among pairs (data not shown). At PN126, sacrifice took place between 9:00 and 12:00 hrs. 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 and centrifuged at 2600G for 10 minutes, and plasma was stored at -80°C until further analyses. Brain regions (hypothalamus), liver and fat pads (perirenal, retroperitoneal, inguinal, epididymal, mesenteric, 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 for one week 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 fat mass to lean mass ratio was calculated by the following formula (total fat mass / body lean mass). Five data points could not be used due to unforeseen technical issues.

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 by van Dijk et Al (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) (Reijne et al., 2016). Four samples for insulin and CORT, and three for glucose could not be analysed due to unforeseen circumstances.

RNA isolation and quantitative real-time PCR

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* 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) 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. 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. The relative gene expression was calculated using the delta delta Ct method using *Gapdh* as housekeeping gene. Samples with either deviating individual PCR efficiency of more than 5%, or baseline errors or noisy samples have been excluded from the calculations. Therefore, 33 samples have been excluded for *Pomc*, 25 for *Lep*, 28 for *NPY*, 16 for *Mc4r*, 17 for *Bdnf*, 11 for *Crh*, 13 for *Ghrh* and 10 for *Somatostatin*.

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
Gapdh	ACAACTTTGGCATTGTGGAA	GATGCAGGGATGATGTTCTG

Table 2. Primer sequences.

Statistical analyses

Sample size was estimated based on our previous experience in carrying out metabolic experiments involving C57BL/6J mice. Overall, sample size was calculated taking into account the number of animals necessary for indirect calorimetry. An average effect size of 0.29 for indirect calorimetry readouts, 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.

Statistical analyses were performed using RStudio 1.3.959. General linear models were constructed to evaluate the effects of the factors housing, temperature and diet on either FCM, body weight gain, BMC, BMD, EI, EE, RMR, FM (g and %), LM (g and %), FM/LM, fat pads weight, femur length and relative gene expression. General linear models were constructed to investigate the effects of the factors diet and social rank in SOC mice only, on FCM, BMC, BMD, BW, FM, LM, FM/LM, fat pads weight and femur length. A general linear mixed-effects model was used to examine whether housing and temperature 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 housing, temperature and diet predicted body weight from PND42 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 semi-fasted compared to all the other timepoints. 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). IBM SPSS Statistics (version 26) was used to run multiple stepwise regressions to predict one dependent variable (either RMR, EI, LM or FM) from temperature, housing, diet and the factors that were not considered as dependent variables (that were independent of the dependent variable). As RMR/EI were dependent from one another, they were not used together in the same models. This led to the six different models: 1) dependent variable: RMR, independent variables: FM, LM, temperature, housing, diet;2) dependent variable: EI, independent variables: FM, LM, temperature, housing, diet; 3) dependent variable: LM, independent variables: FM, EI, temperature, housing, diet; 4) dependent variable: LM, independent variables: FM, RMR, temperature, housing, diet; 5) dependent variable: FM, independent variables: LM, EI, temperature, housing, diet; 6) dependent variable: FM, independent variables: LM, RMR, temperature, housing, diet. Graphical design was performed using the ggplot2 R package and 3D scatterplots with the use of the scatter3d function from the rgl 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

Effect of individual housing, increased environmental temperature and high fat diet on body weight and body weight gain

Groups did not differ in body weight at the beginning of the housing and temperature interventions (PND21). Linear mixed model assessment of housing and temperature effects on body weight from PND21 to PND42 revealed that IND significantly decreased body weight ($p = 0.02$), but temperature did not ($p = 0.58$). The difference in body weight between IND and SOC housed animals was first detected at PND35 ($p = 0.01$) (Figure 2A). These results were also reflected by the adolescence weight gain, where IND gained less weight from PND21 till PND42 ($p < 0.001$) and temperature did not affect this parameter (Figure 2B). Linear mixed model assessment of housing, temperature and diet effects on body weight from PND42 to PND125 revealed that, despite the increased absolute body weight of IND mice at baseline (PND42, $p < 0.001$), IND decreased ($p < 0.001$) and HF-feeding increased ($p < 0.001$) adult body weight. Interestingly, temperature did not affect body weight ($p = 0.9$) (figure 2C). Finally, adult body weight gain of IND was lower than SOC ($p = 0.03$) and a temperature x diet interaction ($p = 0.02$) indicated that the differences in body weight gain between mice fed a HF vs a LF diet were higher at 21°C compared to 28°C (Figure 2D).

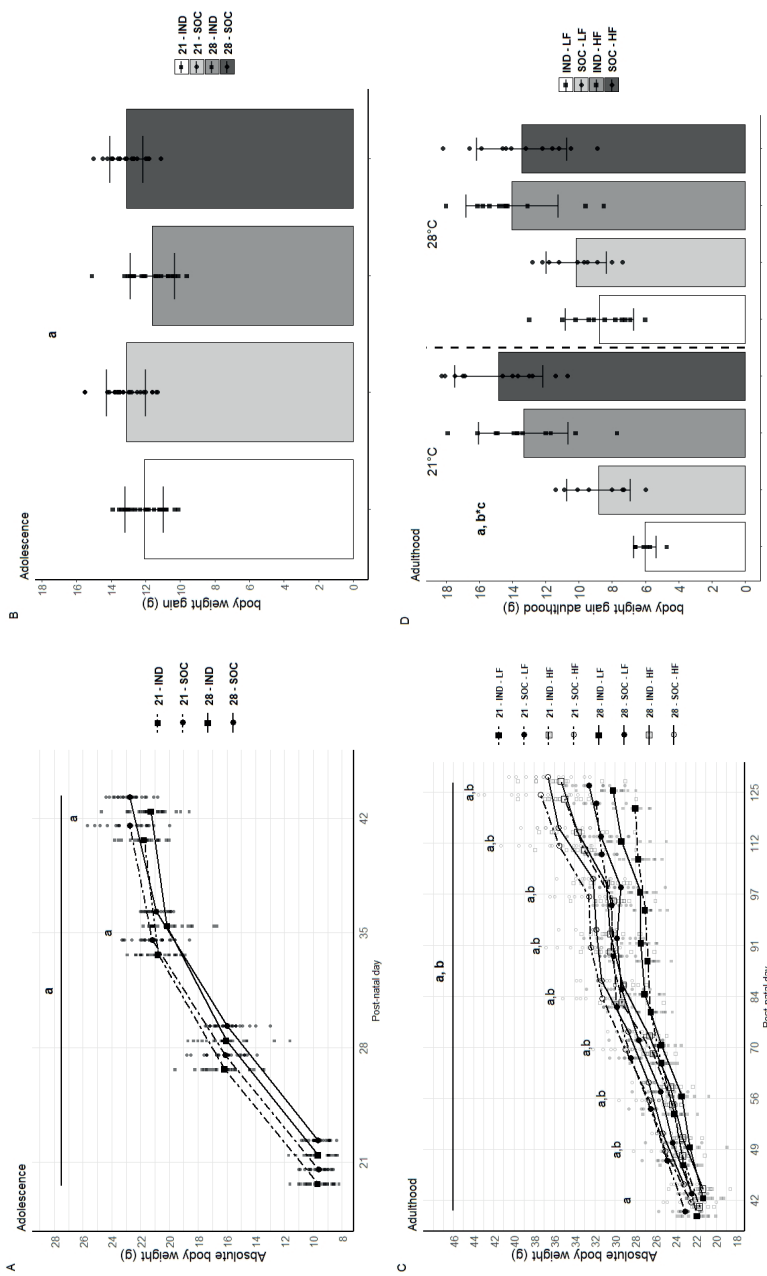

Figure 2. **(A)** Body weight from weaning till PND42 (21-SOC, 21-IND, 28-SOC, 28-IND; n=24 per group). **(B)** Adolescence body weight gain (21-SOC, 21-IND, 28-SOC, 28-IND; n=24 per group). **(C)** Body weight from PND42 till PND125 (21-IND-LF, 21-SOC-LF, 21-IND-HF, 21-SOC-HF, 28-IND-LF, 28-SOC-LF, 28-IND-HF, 28-SOC-HF; n=12 per group; two statistical units missing at PND112: 28-IND-LF n=11 & 28-IND-HF n=11; thirteen statistical units missing at PND125: 21-IND-LF n=7, 21-SOC-LF n=8, 28-IND-LF n=11, 28-SOC-LF n=10, 28-IND-HF n=11). **(D)** Adult body weight gain (21-IND-LF n=7; 21-SOC-LF n=8; 21-IND-HF n=12; 21-SOC-HF n=12; 21-SOC-HF n=12; 28-IND-LF n=11; 28-SOC-LF n=10; 28-IND-HF n=11; 28-SOC-HF n=12). a = significant main effect of housing, b = significant main effect of diet, c = main effect of temperature, b * c = diet x temperature interaction. For figure 2A and 2C, data is represented with individual dot-points, means ± standard deviation (SD). For figure 2B and 2D, data is represented with individual dot-points, means ± standard deviation (SD).

Effect of individual housing, increased environmental temperature on fecal Testosterone metabolites (FCM)

Fecal testosterone metabolite concentrations at PND42 were increased in IND mice compared to SOC mice at PND42 ($p = 0.02$) (Figure 3), whereas temperature did not affect this readout.

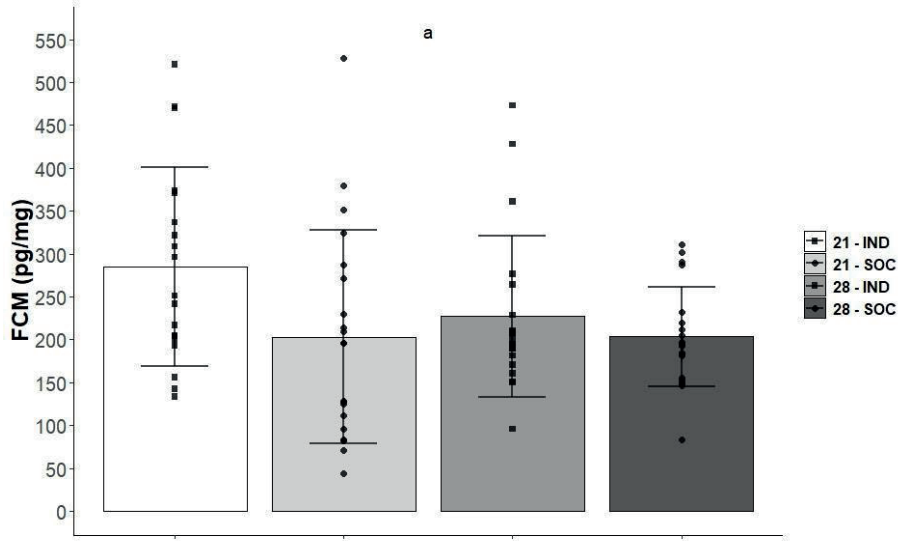

Figure 3. Fecal testosterone metabolites expressed as concentration (pg) per fecal mass (mg). (21-SOC n=20, 21-IND n=19, 28-SOC n=20, 28-IND n=20; one outlier removed from group 21-IND, FCM=724.8), a = significant main effect of housing. Data are individual dot-points, means ± standard deviation (SD).

Effect of individual housing, increased environmental temperature and high fat diet on bone homeostasis assessed by DXA scan (PND97)

Bone composition analysis by DXA showed that BMC was decreased by IND ($p < 0.001$) and increased by HF-feeding ($p < 0.001$) (Figure 4A). BMD was slightly increased in HF-fed mice ($p < 0.001$). There was also an interaction housing x temperature ($p = 0.01$), although Tukey's post-hoc test did not show any significant differences between the groups (Figure 4B).

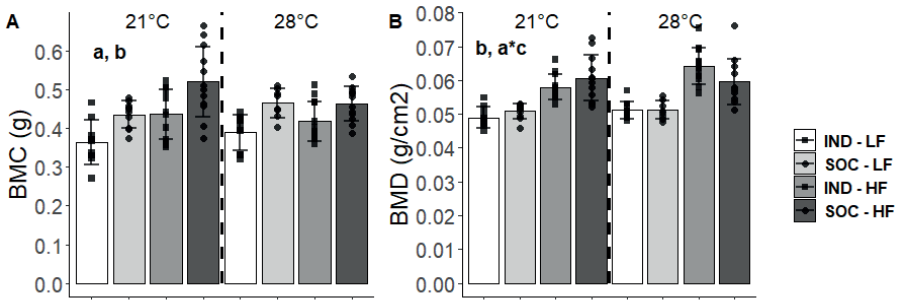

Figure 4. Bone homeostasis assessed by DXA scan. **(A)** Bone mineral content (g) and **(B)** bone mineral density (g/cm²). (21–IND–LF n=9; 21–SOC–LF n=10; 21–IND–HF n=12; 21–SOC– HF n=12; 28–IND–LF n=10; 28–SOC–LF n=8; 28–IND–HF n=12; 28–SOC–HF n=12). Data are individual dot-points, means ± standard deviation (SD). a = significant main effect of housing, b = significant main effect of diet, c = significant effect of temperature, a * c = housing x temperature interaction.

Effect of individual housing, increased environmental temperature and high fat diet on energy intake, energy expenditure and resting metabolic rate assessed in the InCA system

Energy intake (EI) was assessed manually in the InCa system (PND113-119) and was corrected either for body weight or for lean mass. A housing x temperature interaction was present both when EI was normalized per gram of BW ($p = 0.002$, figure 5A) and LM ($p = 0.004$, figure 5B). In particular, Tukey post-hoc test showed that energy intake was higher in IND than SOC only at 21°C and not at 28°C (21°C-IND-LF vs 21°C-SOC-LF $p < 0.001$, figure 1A; 21°C-IND-LF vs 21°C-SOC-LF $p < 0.001$, 21°C-IND-HF vs 21°C-SOC-HF $p = 0.007$, figure 1B). Furthermore, HF feeding increased EI normalized per gram of LM ($p = 0.007$) compared to LF feeding (figure 5B).

Both EE and RMR assessed by InCA showed a housing x temperature interaction both when normalized per BW and LM ($p < 0.001$, figure 5C-F). Tukey post-hoc analysis showed that IND mice had increased EE (KJ/BW), EE (KJ/LM), RMR (KJ/BW) and RMR (KJ/LM) compared to SOC mice, only at 21°C and not at 28°C (figure 5C-F). In addition, a diet effect was present for EE (KJ/BW) ($p < 0.001$) and RMR (KJ/BW) ($p = 0.02$, figure 5C and 5E), indicating that HF-feeding decreased EE and RMR when this was normalized per gram of body weight. Conversely, a housing x diet interaction for EE (KJ/LM) ($p = 0.01$) and RMR (KJ/LM) ($p = 0.006$) was present, denoting that when EE and RMR were corrected per gram of lean mass HF-feeding increased EE and RMR, mostly at 21°C (Figure 4 A-D).

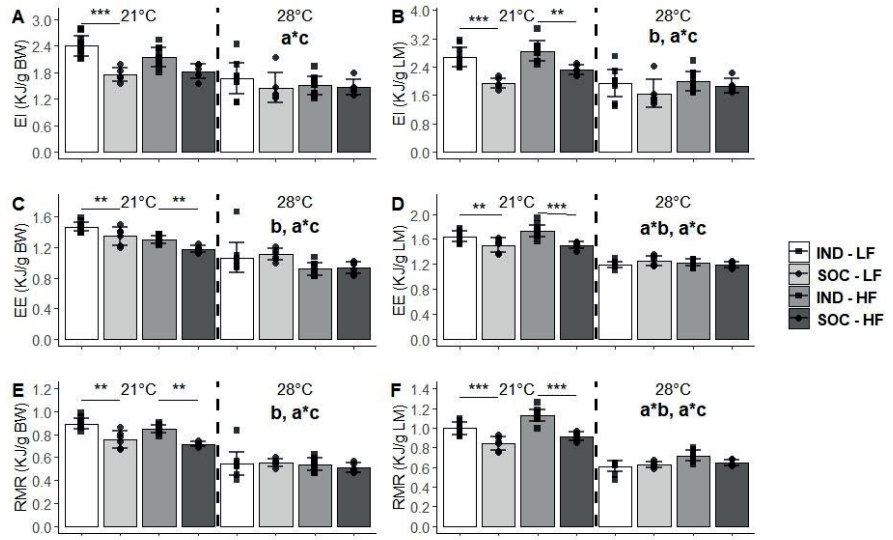

Figure 5. Energy intake (EI), energy expenditure (EE) and resting metabolic rate (RMR) averaged over 24 hours and corrected either per gram of body weight (BW) or lean mass (LM), assessed in an indirect calorimetry system (InCA). **A)** EI (KJ/g BW), **(B)** EI (KJ/g LM), **(C)** EE (KJ/g BW), **(D)** EE (KJ/g LM), **(E)** RMR it (KJ/g BW) and **(F)** (KJ/g LM). For figure A and B, group composition was: 21–IND–LF n=11; 21–SOC–LF n=6; 21–IND–HF n=11; 21–SOC– HF n=6; 28–IND–LF n=12; 28–SOC–LF n=6; 28–IND–HF n=11; 28–SOC–HF n=6. For figures C-F, group composition was: 21–IND–LF n=11; 21–SOC–LF n=6; 21–IND–HF n=12; 21–SOC– HF n=6; 28–IND–LF n=11; 28–SOC–LF n=6; 28–IND–HF n=10; 28–SOC–HF n=6. Data is represented as individual dot-points, means ± standard deviation (SD). a = significant main effect of housing, b = significant main effect of diet, c = significant effect of temperature, a*b = significant housing x diet interaction, a*c = significant housing x temperature interaction.

Effect of individual housing, increased environmental temperature and high fat diet on body composition at PND126

At PND126, 12 weeks after the start of diet exposure and 15 weeks after ambient temperature assignment, body weight of IND was significantly reduced (- 10.5%) compared to SOC ($p < 0.001$) (table 3). Whereas absolute total fat mass was not affected by IND ($p = 0.27$, figure 6A), a significant reduction in lean mass (- 7%, $p < 0.001$, figure 6B) and an increased fat mass to lean mass ratio (+ 19%, $p = 0.001$, figure 6C) were shown for IND mice. In line with this, the proportion of fat in relation to body weight ($p < 0.001$), inguinal ($p = 0.02$) and subcutaneous fat depots ($p = 0.02$) were also increased (table 3). Finally, IND decreased significantly femur length compared to SOC (-2.5%, $p < 0.001$, figure 6D), whereas diet and temperature did not affect this readout.

Carcass analysis showed that mice housed at 28°C had increased absolute total fat (p = 0.045, figure 6A) despite no differences in body weight (p = 0.34, table 3). These changes were reflected in a different proportion of body fat (p = 0.007) and lean body mass (p = 0.02) relative to body weight (table 3) and in increased fat mass to lean mass ratio (p = 0.02, figure 6C). The increased adiposity of the mice living at 28°C was associated with increased perirenal (p = 0.002), epididymal (p = 0.03) and brown adipose tissue (p = 0.004) fat depots (table 3).

Finally, 12 weeks of high fat diet exposure increased body weight (p < 0.001), fat mass (p < 0.001, figure 6A), fat to lean mass ratio (p < 0.001, figure 6C) and all the fat pads weighed at PND126 (see table 3). Interestingly, lean mass was unaffected by HF feeding (p = 0.84, figure 6B).

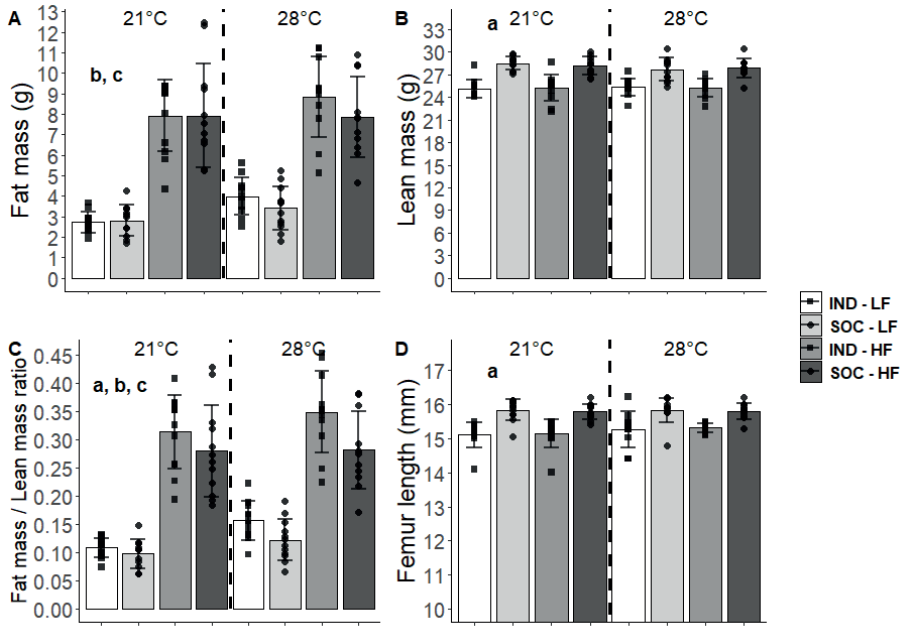

Figure 6. Body composition analysis performed at PND126. **(A)** Fat mass (g), **(B)** lean mass (g), **(C)** fat mass to lean mass ratio and **(D)** femur length (mm). Data is n=12 per group, with five data points missing for fat mass and lean mass and fat mass to lean mass ratio (21–IND–LF n=11; 21–SOC–LF n=11; 21–IND–HF n=12; 21–SOC– HF n=12; 28–IND–LF n=11; 28–SOC–LF n=12; 28–IND–HF n=11; 28–SOC–HF n=11) and four for femur length (21–IND–LF n=11; 21–SOC–LF n=12; 21–IND–HF n=11; 21–SOC– HF n=12; 28–IND–LF n=11; 28–SOC–LF n=12; 28–IND–HF n=11; 28–SOC–HF n=12). Data is represented as individual dot-points, means ± standard deviation (SD). a = significant main effect of housing, b = significant main effect of diet, c = significant effect of temperature.

	21 - IND - LF	21 - SOC - LF	21 - IND - HF	21 - SOC - HF	28 - IND - LF	28 - SOC - LF	28 - IND - HF	28 - SOC - HF
Absolute body weight (g)	27.9 ± 1.6	31.2 ± 1.3	33.1 ± 3	36.1 ± 3.3	29.4 ± 1.7	31.1 ± 2.3	34 ± 2.9	35.8 ± 2.5
Total fat mass (g)	2.7 ± 0.5	2.8 ± 0.8	7.9 ± 1.7	7.9 ± 2.5	4 ± 0.9	3.4 ± 1.1	8.8 ± 2	7.9 ± 2
Total fat mass (%)	9.8 ± 1.4	8.9 ± 2.1	23.7 ± 3.9	21.6 ± 4.8	13.5 ± 2.6	10.8 ± 2.9	25.7 ± 4	21.8 ± 4.1
Body lean mass (g)	25.1 ± 1.2	28.5 ± 0.9	25.2 ± 1.8	28.1 ± 1.2	25.4 ± 1.1	27.7 ± 1.5	25.2 ± 1.2	27.9 ± 1.3
Body lean mass (%)	10.9 ± 1.7	9.8 ± 2.6	31.4 ± 6.6	28 ± 8.1	15.7 ± 3.4	12.2 ± 3.6	34.9 ± 7.2	28.2 ± 6.8
WAT, perirenal (mg)	58.7 ± 18.7	74.1 ± 36.3	162 ± 53.7	184.6 ± 66	95.9 ± 32.1	89.7 ± 36	212.7 ± 57.6	207.2 ± 71.8
WAT, retroperitoneal (mg)	101 ± 29.5	141.5 ± 75.4	487 ± 121	483.5 ± 140.4	200.1 ± 61.8	154.8 ± 79.4	507.9 ± 123.4	462.6 ± 103.8
WAT, inguinal (mg)	274.7 ± 49	257.3 ± 57.8	757.2 ± 223.6	689.9 ± 227.4	377.5 ± 94.4	294.9 ± 101.1	814.7 ± 205.4	644.2 ± 275.6
WAT, epididymal (mg)	426.1 ± 98.5	486.9 ± 137.7	1437.6 ± 346.8	1540.4 ± 426.6	708.3 ± 171.6	587.8 ± 230.4	1623.3 ± 362.4	1513.3 ± 401
WAT, mesenteric (mg)	288.4 ± 71.3	295.2 ± 116	520.3 ± 106.5	610.2 ± 156.6	365.4 ± 136.7	406.4 ± 271.2	588 ± 134	595 ± 160.9
WAT, subcutaneous (mg)	477.1 ± 142.4	378.9 ± 120.7	1366.7 ± 380	1283.8 ± 635.8	575.5 ± 229.2	404.6 ± 146.8	1470.2 ± 454	1150.8 ± 288.8
BAT, interscapular (mg)	158.6 ± 15.1	186 ± 40.1	362.6 ± 85.8	346.5 ± 89.1	222.3 ± 39.8	229.5 ± 57.9	368.4 ± 68.9	397.1 ± 107.3

Table 3. Means ± SD of body weight, fat mass (g & %), lean mass (g & %) and fat pads at sacrifice (PND126). Data is n=12 per group, with three data-points missing for body weight, five for carcass fat mass and carcass lean mass, four for perirenal, retroperitoneal and subcutaneous fat; and three for epididymal, mesenteric and brown adipose tissue. a = significant main effect of housing, b = significant main effect of diet, c = significant effect of temperature.

Effect of individual housing, increased environmental temperature and high fat diet on plasma levels of glucose, insulin, corticosterone, HOMA-IR, triglycerides and leptin

Plasma glucose levels were decreased by IND (p = 0.04) and at 28°C (p = 0.04), whereas they were increased by HF-feeding (p < 0.001). Plasma levels of insulin were decreased significantly by IND (p = 0.01) and unaffected by diet (p = 0.22) and temperature (p = 0.18). The homeostatic model assessment (HOMA-IR) was significantly decreased by IND (p = 0.003) and increased by HF-feeding (p = 0.004). Plasma levels of CORT were unaffected by housing , diet and temperature. Plasma levels of triglyceride were significantly increased by LF feeding (p < 0.001) and unaffected by diet and temperature, whereas plasma leptin levels were significantly increased by HF feeding (p < 0.001) but unaffected by diet and temperature. Graphical representation of plasma measurements is represented in figure 7.

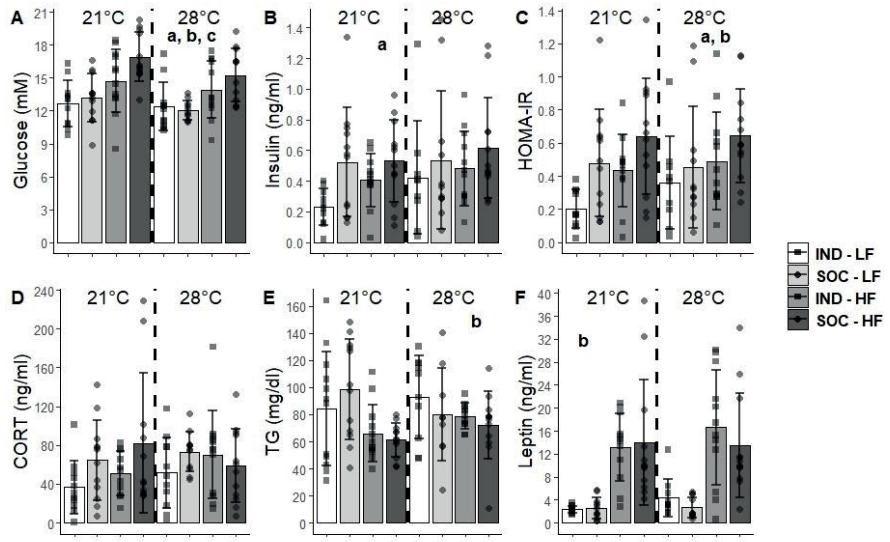

Figure 7. Plasma levels of **(A)** glucose (mM) and **(B)** insulin (ng/ml), **(C)** calculated HOMA-IR, **(D)** plasma corticosterone (ng/ml), **(E)** triglycerides (TG, mg/dl) and **(F)** leptin (ng/ml). Four samples for insulin, CORT and leptin, three for glucose and six for TG could not be analysed due to unforeseen circumstances. Group composition for glucose was the following: 21–IND–LF n=11, 21–SOC–LF n=12, 21–IND–HF n=12, 21–SOC– HF n=12, 28–IND–LF n=11, 28–SOC–LF n=11, 28–IND–HF n=12, 28–SOC–HF n=12. Group composition for insulin was the following: 21–IND–LF n=10, 21–SOC–LF n=12, 21–IND–HF n=11, 21–SOC– HF n=12, 28–IND–LF n=9, 28–SOC–LF n=11; 28–IND–HF n=12; 28–SOC–HF n=12, with three outliers removed (values: 1.89, 1.91, 2.8). Group composition for CORT was the following: 21–IND–LF n=11, 21–SOC–LF n=12, 21–IND–HF n=11, 21–SOC– HF n=11, 28–IND–LF n=11, 28–SOC–LF n=11, 28–IND–HF n=12, 28–SOC–HF n=12, with one outlier removed (value: 626.9). Group composition for HOMA-IR was the same as insulin (three outliers removed with values: 1.75, 2.14, 2.47). Group composition for TG was the following: 21–IND–LF n=12, 21–SOC–LF n=12, 21–IND–HF n=12, 21–SOC– HF n=12, 28–IND–LF n=10, 28–SOC–LF n=9, 28–IND–HF n=11, 28–SOC–HF n=12. Group composition for leptin was the following: 21–IND–LF n=11, 21–SOC–LF n=12, 21–IND–HF n=12, 21–SOC– HF n=12, 28–IND–LF n=10, 28–SOC–LF n=11, 28–IND–HF n=12, 28–SOC–HF n=12. Data are represented as individual dot-points, means ± standard deviation (SD). a = significant main effect of housing, b = significant main effect of diet, c = significant effect of temperature.

RNA isolation and quantitative real-time PCR

General linear models found no effects of housing and temperature on relative *Pomc*, *Lepr*, *Npy*, *Mc4r*, *Bdnf*, *Crh*, *Ghrh* and *Somatostatin* mRNA expression in the hypothalamus. High fat feeding decreased the relative mRNA expression of *Mc4r* (p = 0.012), *Crh* (p = 0.010), *Ghrh* (p = 0.021) and *Somatostatin* (p = 0.026), compared to LF-feeding (figure 8).

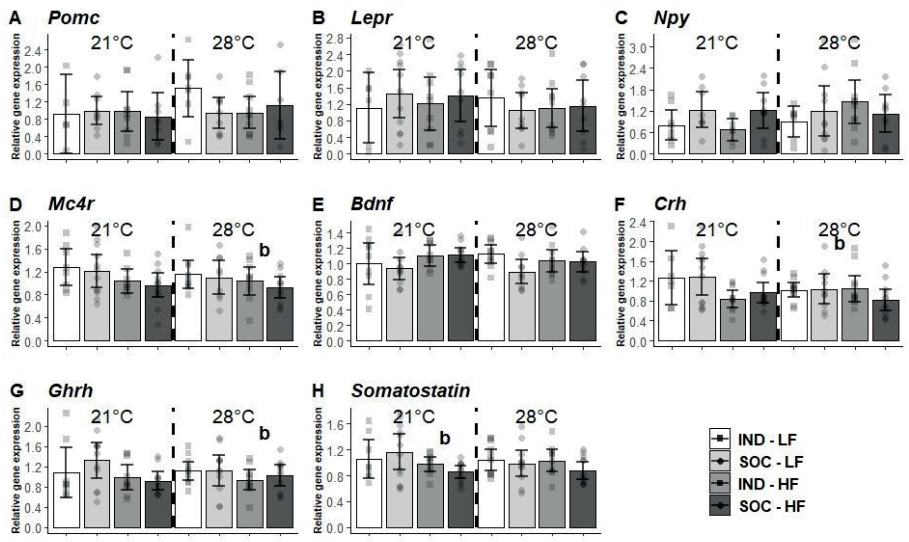

Figure 8. Relative *Pomc*, *Lepr*, *Npy*, *Mc4r*, *Bdnf*, *Crh*, *Ghrh* and *Somatostatin* mRNA expression of IND and SOC mice fed either a LF or HF diet and housed either at 21°C or 28°C. Data is reported as means ± interval confidence (95%) and individual dot-points. In total 33 samples have been excluded for *Pomc*, 25 for *Lep*, 28 for *NPY*, 16 for *Mc4r*, 17 for *Bdnf*, 11 for *Crh*, 13 for *Ghrh* and 10 for *Somatostatin*. The exclusion of these samples was due to either deviating individual PCR efficiency of more than 5%, or baseline errors or noisy samples as assessed by LinRegPCR. b = significant main effect of diet.

Effects of social rank on body composition, bone homeostasis, growth and plasma hormones

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 either diet, body weight, the pair's 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. The average number of wins was 3.77 ± 0.92 (mean ± SD) for dominant animals and 1.23 ± 0.92 for subordinate 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 are discussed, as diet has been thoroughly discussed in the previous section.

Social rank did not affect BMC and BMD at PND97 and body weight, fat mass (g), lean mass (g), fat to lean mass ratio, fat pads weight, femur length, plasma levels of glucose, insulin, CORT and HOMA-IR, TG and leptin at PND126 (means showed in table 4).

	Dominant - LF	Subordinate - LF	Dominant - HF	Subordinate - HF
Absolute body weight (g)	31.9 ± 1.5	30.9 ± 1.6	36.3 ± 2.9	35.6 ± 3
Total fat mass (g)	3.4 ± 1	2.8 ± 0.8	8.2 ± 2.3	7.6 ± 2.2
Body lean mass (g)	28.6 ± 1	28.1 ± 1.1	28 ± 0.8	28 ± 1.5
Fat mass / Lean Mass ratio	0.1 ± 0	0.1 ± 0	0.3 ± 0.1	0.3 ± 0.1
WAT, perirenal (mg)	93.5 ± 33.1	74.5 ± 37.3	206.3 ± 78.9	185.6 ± 57.8
WAT, retroperitoneal (mg)	170.6 ± 65.6	126.9 ± 77	479.1 ± 122.9	467 ± 124.6
WAT, inguinal (mg)	313.9 ± 88.9	260.1 ± 75.3	649.2 ± 273.1	684.9 ± 231.3
WAT, epididymal (mg)	583.8 ± 188.2	461.5 ± 139.4	1609.7 ± 416.4	1444 ± 393.4
WAT, mesenteric (mg)	364.4 ± 88.4	282 ± 132.8	629.6 ± 170.8	575.5 ± 140.6
WAT, subcutaneous (mg)	425 ± 131.4	347.8 ± 134.4	1287.8 ± 413	1158.1 ± 570.6
BAT, interscapular (mg)	229.9 ± 66.2	195.6 ± 40.1	386 ± 104.9	357.6 ± 97.1
BMC (g)	0.46 ± 0.04	0.44 ± 0.03	0.49 ± 0.09	0.49 ± 0.06
BMD (g/cm2)	0.052 ± 0.003	0.05 ± 0.002	0.061 ± 0.007	0.059 ± 0.006
Femur length (mm)	15.8 ± 0.1	15.8 ± 0.5	15.7 ± 0.2	15.9 ± 0.2
Glucose (mM)	13.1 ± 2.3	12.4 ± 1.2	15.6 ± 2.4	16.5 ± 2.5
Insulin (ng/ml)	0.58 ± 0.33	0.44 ± 0.38	0.49 ± 0.26	0.66 ± 0.32

Table 4. Means ± SD of body weight, fat mass, lean mass, fat pads, femur length and plasma hormones at sacrifice at PND126 and BMC/BMD at PND97. As temperature did not affect any of the aforementioned parameters, in this table data has been expressed based on social status and diet only.

Multiple regression models to study the effects of housing, temperature and diet on lean body mass, fat mass and resting metabolic rate/energy intake

To investigate which factors could explain the variation of the primary outcomes RMR, EI, FM and LM, multiple linear regression models were tested with one of the aforementioned factors as dependent variable, and with "temperature", "housing", "diet" (and the factors that were not considered as dependent variables) as independent variables. As we found that RMR and EI were dependent of

Dependent variable	Independent variables	Main effect	P-value	Adjusted R^2
RMR	FM, LM, temperature, housing, diet	Temperature FM	$p < 0.001$	0.817 + 0.67
EI	FM, LM, temperature, housing, diet	Temperature FM Housing LM	$p < 0.001$	0.817 + 0.058 + 0.034 + 0.057
LM	FM, EI, temperature, housing, diet	Housing EI Temperature	$p < 0.001$	0.557 + 0.041 + 0.028
LM	FM, RMR, temperature, housing, diet	Housing	$p < 0.001$	0.547
FM	LM, EI, temperature, housing, diet	Diet Temperature	$p < 0.001$	0.728 + 0.02
FM	LM, RMR, temperature, housing, diet	Diet Temperature RMR	$p < 0.001$	0.728 + 0.02 + 0.019

Table 5. Description, main effects, p-values and adjusted R squared of each multiple regression model used to investigate the primary outcomes RMR, EI, FM and LM with the independent variables FM, LM, EI, RMR.

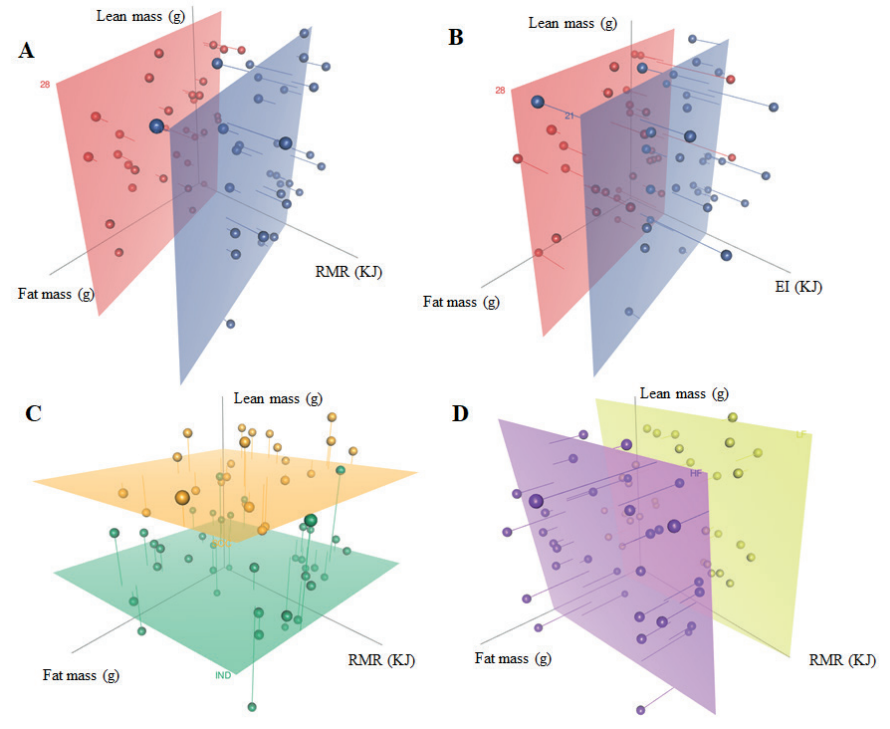

Figure 9. 3D scatterplots representing four of the multiple stepwise regression models. (**A**) Effects that temperature exerted on RMR (blue: 21°C and red: 28°C); (**B**) Effects that temperature exerted on EI (blue: 21°C and red: 28°C); (**C**) effects of housing on lean mass (green: IND and orange: SOC), (**D**) effects of diet on fat mass (yellow: LF and purple: HF).

each other, they were not tested against each other within each model. Overall, we found a strong effect of temperature in predicting RMR and EI, of housing in predicting LM and of diet in predicting FM. The aforementioned models are reported in table 5 and graphically represented in figure 9.

Discussion

In the current study we showed that IND housed mice (compared to SOC housed mice) had increased body fat percentage as well as fat mass content expressed per lean mass at PND 126 irrespective of whether they were housed at RT or at thermoneutrality. This outcome does not appear to be in line with our hypothesis stating that IND housing at thermoneutrality would reduce the need for social thermoregulation, thereby minimizing some of the metabolic consequences of IND housing. The validity of our experimental design appeared nevertheless solid, because resting metabolic rate (RMR: i.e., lowest metabolic rate at a time when mice would be huddling the most, expressed per bodyweight or per lean mass) was higher in IND housed mice than in SOC housed mice at RT, but not at thermoneutrality. The same was found for energy intake (EI), which was increased in IND housed mice relative to SOC housed mice at RT, but not at thermoneutrality. An interesting observation was that the type of diet appeared to have a relatively minor role in abovementioned thermoregulatory findings. Thus, while feeding a HF diet relative to LF feeding clearly augmented body fat (i.e., as percentage as well as expressed per lean mass), increased HOMA-IR (i.e., a proxy for insulin resistance, which is a frequently observed correlate of HF-induced weight gain) and increased leptin levels, it hardly affected the differences in either RMR or EI between IND versus SOC housed mice. If an increase in body adiposity (e.g., due to insulating properties of body fat) would for example limit heat loss, then such an interaction of diet with this discrepancy would be expected too. Our findings are largely in line with the work of Fisher and colleagues, who found no insulating role for body fat in limiting heat loss (Fischer et al., 2016).

Beside a role of social housing condition in regulation of energy intake and expenditure at RT, we observed that IND housed mice had reduced lean mass as well as reduced femur length compared to SOC irrespective of diet type, and this is well in line with our previous findings (Schipper 2020). Here we additionally show that this effect is also independent of whether the mice are maintained at RT or thermoneutrality. Since lean mass strongly contributes to RMR, this finding suggests that the excess RMR at RT in IND housed mice relative to SOC housed

mice could be the result of mechanism unrelated to differences in lean mass, most likely by increased activity of uncoupling protein 1 (UCP-1) in brown adipose tissue or beige white adipose tissue (Schipper 2020; Schipper et al., 2018). Although UCP-1 expression was not determined in WAT or BAT in the current study, it was found to be elevated as a result of IND at RT in our previous study (Schipper 2020). Induction of UCP-1 may also interact with glucose homeostasis as previously shown by Wang et al. (Wang et al., 2015) and Clayton and McCurdy (Clayton & McCurdy, 2018), which potentially may explain the seemingly discordant findings in the present study that IND housed mice had a lower HOMA-IR with higher fat and lower lean mass than those that were SOC housed. As mentioned above, differences in HOMA-IR were in line with the reported effects of HF feeding to increase insulin resistance (van Dijk et al., 2013), thus ensuring the validity of this measure in the present study.

IND housing persistently reduced lean mass, femur length and reduced bone mineral content, which may be explained by several underlying mechanisms. During adolescence, a rapid growth spurt in mice is characterized by a rapid increase in lean mass and bone mineral content (Gargiulo et al. 2014; Malik 1984). This process is also associated with increased energy demands, compared to adulthood maintenance requirements (Nutrition 1995). Differences in energy intake and energy expenditure during this period can directly affect the growth rate of IND mice, and may explain the observed reduced weight gain of IND mice between PND21 and PND42. Another factor that may impair (skeletal) growth is the absence of physical activity related to social behaviours in IND mice - such as playing behaviour and fighting – as these may induce increased mechanical loading, therefore SOC mice may have longer bones and increased bone mineral content (Meakin et al. 2013). Androgens such as testosterone are fundamental for bone formation and stimulation of growth of lean mass at adolescence (Venken et al. 2007). It has been suggested that winning fights elevates plasma testosterone levels in male mice (Oyegbile and Marler 2005) and we hypothesized that social behaviours may be responsible for increased levels of testosterone. However, fecal testosterone metabolite analysis at PND42 indicated that IND mice had increased levels of these metabolites, suggesting that other factors could be influencing growth and development. Growth hormone (GH) is another factor that contributes to the development of bone and lean mass in adolescence (Ohlsson et al. 1998). GH is released by the anterior pituitary gland by GH-releasing hormone (GHRH) and somatostatin (Lin-Su and Wajnrajch 2002). At adulthood, gene expression analysis of *Ghrh* and *Somatostatin* however showed no differences between IND and SOC housed mice, however we did not investigate plasma GH levels. A

limitation of these findings is that gene expression analysis has been performed at PN126, that is beyond adolescence and eventual differences in key regulatory gene expression may have appeared earlier. The only changes in hypothalamic gene expression that we did observe were related to the diet, with reductions in *Mc4r*, *Crh*, *Ghrh* and *Somatostatin* in mice subjected to the HF diet. Changes in these hypothalamic factors could be relevant for the increased storage capacity of adipose tissue in the HF fed mice, without affecting their lean mass (Girardet & Butler, 2013; Baldini & Phelan, 2019; Auvinen et al, 2012; Pérez-Romero et al., 1999).

In the present study, mice were exposed to SOC versus IND housing from weaning onwards, and we observed a reduced growth rate in the latter ones irrespective of ambient temperature. These findings are in contrast with those of Toth et al, who found no changes in body weight in mice with housing density varying from 5 to 1 mice, although in that study the housing intervention started at 8 weeks of age and not at weaning (Toth, Trammell, and Ilsley-Woods 2015). In another study, post-weaning IND resulted in no differences in body weight versus group housing in adolescent C57BL/6J mice (Bibancos et al. 2007; Lopez and Laber 2015) whereas it did increase body weight at 7 and 11 weeks of age in C57BL/6J mice (Tsuduki et al. 2015). Variations in design and experimental conditions between these studies could influence body weight trajectories in IND versus SOC mice and could explain the high heterogeneity found between studies reporting body weight in IND versus SOC rodents (Schipper et al. 2018). Among the factors that could influence body weight is the cage-density of the social-housed groups. We chose to pair housed male mice with two littermates to decrease the risk of fighting between cage-mates and to decrease litter size variation (Bartolomucci et al., 2001), meanwhile the studies aforementioned housed together 4 – 6 unfamiliar male mice. It is well-known that at increased cage densities an increased variation can arise (Nicholson et al. 2009), as fighting and the development of complex social hierarchies can affect phenotypic variation (Varholick et al. 2019). In the current study, clear dominance hierarchies were observed in the SOC housed mice, but these did not pertain to clear dominant-subordinate phenotypes, like changes in the activity of the HPA axis (including hypothalamic CRH expression and plasma corticosterone levels) as mentioned before by others (Pohorecky, Baumann & Benjamin, 2004).

In summary, the present study showed that thermoneutrality did not ameliorate the effects of IND housing to alter certain energy balance characteristics that were previously reported for housing at RT. A much debated question is which is the best temperature to house mice in order to best mimic human physiology and disease, as it is quite accepted that room temperature induces mild cold stress in mice (Keijer, Li, and Speakman 2019; Speakman and Keijer 2013). Cold stress has been proposed as a limit for the translational value of mice models in this respect (Karp 2012; Ganeshan and Chawla 2017; Hankenson et al. 2018). What we found, however, is that maintenance of mice at 21°C or 28°C was by far the strongest factor to regulate energy fluxes RMR and EI, irrespective of whether the mice were fed a HF diet or LF diet, or whether they were IND or SOC housed. This was also the key finding that emerged from the linear regression analysis, with ambient temperature, housing and diet (and some energy balance factors like adiposity and lean mass) as dependent factors. Indeed, the strength of our study was that it allowed us to compare the relative contribution of these conditions in a full factorial design. Besides the relevance of ambient temperature for regulation of energy fluxes, it also became clear that diet was the prime factor relating to adiposity (with HF diet causing more adiposity than LF diet), and the social housing condition was the prime factor relating to lean mass (with SOC housing causing more lean mass than IND housing). Further studies in mice may benefit from these insights by choosing conditions for optimal study of mechanisms in biomedicine research.

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