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

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Chapter 6

General discussion

The use of mouse models has been of tremendous value to understand the mechanisms regulating energy balance regulation, as they share characteristics of human obesity (Lutz & Woods, 2012). The C57BL/6J mouse has been extensively used in this respect, as it is prone to obesity and metabolic dysregulations (Collins et al., 2004). However, there are some limitations to bear in mind when using mouse models to study energy balance regulation and obesity, as common experimental procedures and environmental conditions can affect experimental reproducibility and the translation of findings using mouse models of obesity (Kleinert et al., 2018). Among the factors that can affect experimental outcomes, the number of mice present in a litter or a cage, and the temperature at which mice are kept can strongly affect growth, metabolic health and the translatability of mouse models to humans and be a possible moderator of reported outcomes (Schipper et al., 2018; Parra-Vargas et al., 2020; Speakman & Keijer., 2013). Therefore, a better understanding of these factors is important. The overall aim of this thesis was to study the effects of various social (number of pups in a litter; individual versus social housing at weaning) and environmental factors (housing temperature) on growth and energy balance regulation, using C57BL/6J mice exposed to either healthy or obesogenic diets. In addition to investigating social and environmental factors, a part of this thesis focuses on improving methodology for studying feeding behaviour and meal related parameters in mice. Indeed, mouse models are widely employed for studying feeding behaviour and meal-related parameters as well (Ellacot et al., 2010), and current methodologies to obtain a criterion to define a meal in mice are scant.

Summary of the results

In **chapter 2**, previously published literature was used to investigate the neuroendocrine mechanisms resulting from postnatal overfeeding. In rodents, postnatal overfeeding can be induced by decreasing the size of the litter of the dam during the lactation period, resulting in an increased milk energy transfer from mother to the individual pup. Mice and rats raised in small litters were found to be hyperphagic and become heavier than control rodents as early as during lactation; with these effects persisting into adulthood also in relatively healthy (low fat diet) conditions. These changes were associated with early life hyperleptinemia and hyperinsulinemia and by disturbances in fuel homeostasis; all traits associated with obesity. Hyperleptinemia is a consequence of the increased fat mass deposition shown by rodents raised in small litters. The mechanisms that may play a role in the development of hyperinsulinemia are likely to be in the pancreas, adipose tissue and the liver, where glucose transport is deranged and as a consequence insulin secretion can be increased. Interestingly, early life hyperleptinemia and hyperinsulinemia likely affect the development of the hypothalamus, leading to hyperphagia and body weight gain and associated disturbances in fuel homeostasis. I concluded that litter size reduction is a suitable model to mimic postnatal overfeeding and to study the resultant neuroendocrine, physiological and functional adaptations. However, there are some aspects to consider when applying this model. Indeed, a high level of variation seems to be present between studies and this may be the result of strain differences, litter gender composition, number of pups assigned to control litter size, and litter-sex distribution, which all have effects on the pups and possible outcomes.

In **chapter 3**, the effects of postweaning individual housing relative to social housing on growth, energy balance regulation and metabolic health were investigated both at standard room temperature (21°C) and at thermoneutrality (28°C), either in low fat and high fat conditions. The major outcome of this study was that postweaning individual housing induced a dramatic reduction in body weight, lean mass and femur length at adulthood, indicating a strong effect of housing on growth and energy balance regulation. At the same time, individually housed mice presented more body fat percentage as well as fat mass content expressed per lean mass, suggesting an increased susceptibility to obesity. Importantly, individual housing presented these changes both at 21°C and at 28°C, although at 21°C individually housed mice had increased energy expenditure and energy intake than socially housed mice. Increased environmental temperature decreased both energy intake and expenditure, but did not lead to considerable changes in metabolic health and body composition. Finally, a high fat diet that was provided to induce a metabolic challenge, led to increased fat mass and worsened metabolic health, leaving lean mass unaffected. As an exploratory parameter, we assessed the establishment of social hierarchical structures by the means of a tube test in social housed mice. Interestingly, clear dominance hierarchies were observed in socially housed male mice, but social status did not appear to affect the (variability) in metabolic health outcomes that were investigated in the current study. It was concluded that postweaning individual housing versus social housing is a strong determinant of growth and energy storage, irrespective of environmental temperature and diet. On the other hand, temperature was the strongest factor regulating energy fluxes and diet was the prime factor regulating adiposity.

In **chapter 4**, the effects of postweaning individual housing on growth, obesity development and metabolic health were studied in female mice, relative

to social housing. The effects on growth, body composition and metabolic health of individual versus social housing were relatively small. The main factor regulating energy balance in female mice was the exposure to a high fat diet. When including hierarchical status of socially housed animals as exploratory analysis, social hierarchies in female mice influenced energy balance regulation. In particular, dominant female mice fed a high fat diet showed increased body weights compared to subordinate mice fed the same diet. Furthermore, dominant females showed an increase in lean mass and fat mass and an increase in plasmatic leptin levels irrespective of diet type. I concluded that the effects of individual housing versus social housing in female mice are relatively small, but within-cage differences in energy balance regulation on the basis of dominance hierarchy, however, were found to be quite striking.

In feeding behaviour studies using mice, current methodologies to obtain a criterion to define a meal are scant and researchers often determine arbitrarily a criterion for meal definition, without being aware if this practice can affect meal related parameters. In **chapter 5**, a novel methodology was developed and validated to assess meal definition in mice, using increasing intermeal intervals as a function of averaged meal size. The same male mice housed individually described in chapter 3 were used for this purpose, minimising the number of mice and allowing us to test for the same experimental variables of chapter 3 (diet and temperature). For each animal, each given intermeal interval was taken as a hypothetical meal definition parameter and the resultant average meal size was calculated. This was done by clustering feeding bouts separated by intervals shorter than the intermeal interval considered, whereas they were regarded as separated meals when separating intervals were longer. The relationship between average meal sizes and minimal intermeal intervals followed a broken-stick model and individual breakpoints were assessed automatically. This way, individual breakpoints were used as meal definition criterions. Importantly, breakpoints were significantly shorter in mice fed a high fat diet and longer in mice kept at 28°C, suggesting that using standard arbitrary intermeal intervals (i.e. the use of the same intermeal interval to define meals for each animals, irrespective of diet and temperature) may potentially alter meal related parameters, as the effects of environmental temperature and diet on breakpoint would be missed. The comparison of meal related parameters obtained with either this method or arbitrary intermeal intervals (i.e. 5, 10, 15, 20 minutes) revealed that the choice between these models affect meal related parameters and the main effects of environmental temperature and diet. In fact, meal size, meal duration and average intermeal intervals separating meals were increased with increasing length of

intermeal chosen, and meal frequency and ingestion rate that decreased. Most importantly, the effects of environmental temperature and diet on meal related parameters were dependent on the arbitrary intermeal interval chosen, indicating that the choice of such arbitrary intermeal intervals can obscure the effects of metabolic and dietary manipulations. It was concluded that breakpoint analysis is possible and valid on individual data sets of food intake patterns obtained from mice over a 5 day interval and more awareness should be raised on the use of arbitrary intermeal intervals to define meals.

Discussion

In this thesis, it has been shown that early life (social) manipulations of the number of pups or young mice present in a litter or in a cage can affect growth, energy balance regulation and metabolic health. This is particularly relevant for the interpretation and the translation of results obtained from rodent experiments, as preclinical studies are often used for the design of social and other environmental conditions. Therefore, a careful evaluation of the social and environmental factors studied in this thesis on growth and energy balance regulation is advised.

In **chapter 2** we showed that the number of pups present in a litter can greatly affect energy balance regulation and metabolic health of the animals. In particular, rodents raised in small litters develop consistently weight gain and obesity between studies and these changes are often associated with worsened metabolic health. These effects are likely the result of the increased milk energy transfer from mother to the individual pup, but also other effects cannot be ruled out. Indeed, a different number of pups huddling in a litter can affect heat loss and energy expenditure (Stefanidis & Spencer, 2012). Besides this limitation and the factors to take into account when applying the litter size reduction (strain differences, litter gender composition, number of pups assigned to control litter size, and litter-sex distribution), this model seems a good strategy to investigate postnatal overfeeding. This is relevant, as bottle feeding (i.e. an alternative to children unable to receive maternal milk) could make infants vulnerable to postnatal overfeeding (Appleton et Al., 2018) and this is a practice that can increase early life weight trajectories (Weng et al., 2012) and is associated with susceptibility to overweight, obesity and related comorbidities later in life (Hopkins et al., 2015; Singhal et al., 2010). On the other hand, it is important to be aware of the effects that litter size can have on energy balance regulation and metabolic health, and information on litter sizes should be always reported in energy balance studies

(Parra-Vargas et al., 2020) for a better interpretation and translation of animal experiments to the human situation.

Interestingly, not only the number of pups present in a litter can affect growth, energy balance regulation and metabolic health. Indeed, as shown in **chapter 3,** housing male mice either individually or socially (i.e. in pairs with a littermate) from weaning on can also affect these parameters. In particular, male mice that were individually housed at weaning showed a dramatic reduction in body weight, lean mass and femur length. These changes were accompanied by an increased percentage of fat mass and fat mass to lean mass ratio, suggesting that postweaning individual housing predisposes for obesity. However, the metabolic health of individually housed mice was not worsened as a fatter phenotype may suggest, as shown by an improved glucose homeostasis and insulin sensitivity. This is particularly relevant, since with the litter size reduction model the amount of energy that each individual pup in a small litter obtains is greater than the amount of energy that pups obtain from normal (control) litters. Contrary to the litter size reduction model, in **chapter 3** the animals were fed ad libitum and raised in standard litters of 6 pups each, therefore there was not a factor limiting energy intake between individual and social housed mice. Therefore, differences in energy intake and energy expenditure between individual and socially housed animals can be argued to be the result of either emotional stress or from the lack of social thermoregulation in individually housed mice (Schipper 2018). The latter is particularly important, as individually housed mice do not have the ability to huddle and share body heat, a strategy applied by rodents to limit heat loss (Ebensperger, 2001) and individually housed mice seem to increase energy expenditure and intake as a result of increased thermoregulation (Schipper et al., 2020). To rule out the effects of social thermoregulation on energy fluxes in individually housed mice at room temperature (21°C), we kept half of the mice near their thermoneutral zone (28°C): Interestingly, we found that the effects of postweaning individual housing were independent of environmental temperature, despite energy expenditure and intake were elevated in individually housed mice only at 21°C. This may explain why metabolic health was improved in individually housed mice, as glucose homeostasis can be improved by cold exposure, mainly through the activation of uncoupling-protein-1 (UCP-1) in the brown adipose tissue and "browning" of white adipose tissue (Wang et al., 2015). Although we did not investigate this in **chapter 3**, in our previous investigation, we indeed showed that UCP1, together with energy expenditure were elevated in individually housed mice at 21°C (Schipper et al., 2020).

To understand which mechanisms could be implicated in affecting growth and body composition, we investigated hypothalamic gene expression of genes related to energy metabolisms, stress and growth. These analyses did not suggest that any of the selected genes were affected by housing. However, these measurements were performed in hypothalamic slices obtained at postnatal day 126, that is beyond puberty and adolescence in mice (Brust, Schindler & Lewejohann, 2015). Therefore, possible differences in energy intake, expenditure, stress and growth that may have arisen during adolescence may have not been detected at such late stages. Androgens such as testosterone are fundamental for bone formation and stimulation of growth of lean mass at adolescence (Venken et al. 2007). Investigation of fecal testosterone metabolite at PND42 (adolescence), indicated that testosterone metabolites were elevated in individually housed mice. Contrary to our findings, increased levels of testosterone would accelerate growth trajectories, therefore other mechanisms may have affected growth at this stage. For example, measurement of hypothalamic-pituitary-adrenal axis activity and corticosterone levels during adolescence would have shed some light on whether these mechanisms could have been responsible for the different growth trajectories presented by individually and socially housed mice. Indeed, corticosterone administration in adolescence can decrease adolescent body weight gain, increase fat mass, decrease lean mass, lower plasma glucose and affect skeletal development (Kinlein et al., 2017), all features that have been displayed by individually housed male mice.

Another finding from **chapter 3** is that increasing environmental temperature resulted in a decreased energy expenditure that was compensated for by a decreased energy intake, with no apparent effects on body composition and metabolic health. This is relevant in spite of the fact that mice kept at standard room temperature spend an increased amount of energy to keep warm, and this has been proposed to limit the translational value of mice models for human physiology and disease (Karp 2012; Ganeshan and Chawla 2017; Hankenson et al. 2018). Another factor that arose in **chapter 3** is that mice on a high fat diet displayed increased energy expenditure and resting metabolic rate (when these were normalized per gram of lean mass). In the human literature, it has been suggested that the health consequences of obesity are metabolically costly and therefore they could increase energy expenditure (Soares, Cummings & Ping-Delfos, 2011). Such mechanisms are in line with the findings of Vijgen et al. that showed that obese humans rely less on thermogenesis in cold conditions than lean individuals (Vijgen et al., 2011).

In an exploratory analysis in **chapter 3** it was observed that pair housing male littermates led to clear dominance structures, but did not pertain to metabolic phenotype based on the parameters evaluated in this analysis, like changes in the activity of the HPA axis and body composition. Dominance structures have been reported to contribute to phenotypic variation within cages of laboratory mice (Varholick et al., 2019). However, our findings are in line with Bartolomucci et al. who found that housing together male littermates at weaning does not contribute to variation within a cage (Bartolomucci et al., 2001). This suggests that housing familiar animals together at weaning could be a better strategy than housing unfamiliar animals, since unfamiliar animals tend to fight more and show more aggression towards their cagemates (Kareem & Barnard, 1982; Rowe & Redfern, 1969) and this strategy seems to be even improved if applied at an early age (Zidar et al., 2019). In conclusion, it is important to interpret these findings with caution, as this was an exploratory analysis and it was not certainly powered for drawing conclusions. Future studies should increase sample size and evaluate the effects of social hierarchies also in non-littermate pairs, but also take into account larger groups (more than two animals per cage, either of littermates and unfamiliar animals) to increase awareness on the effects of social hierarchy on energy balance regulation.

In conclusion, with a full factorial design we found that (social) housing conditions were the prime factor that affected lean mass and bone development, whereas environmental temperature affected energy fluxes and diet was the main factor affecting adiposity.

Contrary to chapter 3, using female mice in **chapter 4** we showed that individual housing did not significantly affect growth, body composition and metabolic health relative to social housing. The only differences between the housing conditions were found in lean mass accrual, as individual housed females had a relatively small increase in lean mass compared to social housing and a limited effect on bone mineral density. The difference between these two studies could be explained by potential sex-differences in body weight development, growth and metabolic health following metabolic challenges (Jacobs et al., 2019; Bergmann et al., 1995). Indeed, female mice seem to be more resistant to diet induced obesity and are less vulnerable to metabolic alterations than male mice (Hwang et al., 2010), probably as a result of estrogens (Hong et al., 2009; Stubbins et al., 2012). This is one of the reasons why female rodents are used less than male rodents in studies investigating energy balance and metabolic health, as male rodents develop a more profound obesity and deterioration of metabolic health following

high fat feeding (Mauvais-Jarvis, Arnold & Reue, 2017). Our findings showed that female mice developed obesity following high fat feeding, but with the settings and the readouts chosen, metabolic health was not affected. However, individual versus social housing did not affect growth, body composition and metabolic health as it did in male mice. These findings are relevant and should be taken into account in designing and interpreting studies investigating energy balance using male and female mice. This suggests that experimental designs need to be optimized for each sex separately and findings from one sex cannot be simply extrapolated to the other.

In an exploratory analysis, the establishment of social dominance relationships in the social group and whether these could have an effect on energy balance regulation was investigated. Dominant female mice were heavier than subordinates when fed a high fat diet. In addition, dominant female mice showed increased fat mass, lean mass and leptin levels compared to subordinate cagemates. These differences could have been explained by differences in energy intake and expenditure between cagemates, but unfortunately we were unable to assess these parameters at the individual level. A potential limitation of this study was that social dominance relationships were investigated only at a late stage, as we did not want to potentially inflate rivalry between the females, which subsequently would affect health, weight gain trajectories of socially housed females (relative to individually housed ones). Socially housed female mice were housed together from postnatal day 2, when litter randomization took place and were kept together at weaning (littermates). It remains to be seen whether the establishment of social hierarchies is stable in small groups of littermates in female mice. While the development and dynamics of the dominance hierarchy in the litter or shortly after weaning were not evaluated in the current study, there was already an increase in body weight of the dominant females above the subordinate ones at postnatal day 42, just before they were exposed to the high fat diet and these effects persisted till the end of the experiment. This may suggest that dominance hierarchy was already established at an early age, however more research is needed to unravel the mechanisms of dominance hierarchy establishment in mice. These findings are relevant, but should be interpreted with caution, as this was an exploratory analysis and it was not certainly powered for drawing conclusions. Future research should focus on studying the establishment of social dominance relationships in female littermates housed in pairs and in bigger groups, with a bigger sample size than our experiment. In addition, the measurement of the estrous cycle in female mice should be regularly evaluated, as this seems to be compromised in bigger groups compared to pair-housing in

female mice (Mauvais-Jarvis, Arnold & Reue, 2017) and may potentially influence outcomes in the tube test. Another suggestion for future research is that energy intake should be evaluated at the individual level in socially housed female mice. This would help to unravel potential differences in food intake between dominant and subordinate animals and therefore it may explain some potential differences in energy balance regulation. In conclusion, the results of our experiment suggest that differences in energy balance regulation between individually housed and socially housed littermates are relatively small, certainly in comparison to the impact of feeding a high fat diet versus a low fat diet. In-cage differences in energy balance regulation on the basis of dominance hierarchy, however, were found to be quite striking. However, these findings could not be conclusive, as a small sample was used and social hierarchies were assessed only at the end of the experiment. 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.

In **chapter 5** I showed that the detection of a criterion for meal definition can be done in an automated way, under different experimental and environmental conditions. The feeding behaviour of animals, such as cows, pigs, mice and rats, has been investigated extensively and the determination of a criterion for meal definition (intermeal interval) has been the subject of intense debate within the scientific community. Typically, an intermeal interval for meal definition is found with the use of mixed distribution models (Yeates et al., 2001), log-survivor (Slater & Lester, 1982), log-frequency functions (Sibly, 1990) or drinking-implicit intervals (Zorilla et al, 2005). However, these methodologies take only the length and the frequency of intermeal intervals separating feeding/drinking bouts into account. It has been suggested that meals would be a more relevant unit of animal feeding behaviour (Sibly, 1990; Demaria-Pesce & Nicolaïdis, 1998; Yeates et al., 2001), therefore, a methodology that would take into account how meal related parameters evolve with increasing intermeal intervals and investigate their relationships would make sense in this respect. In a recent paper, Rathod and Di Fulvio applied these concepts in group-housed mice, investigating how meal size and meal frequency develop with increasing (arbitrary) intermeal intervals ranging from 1 to 30 minutes (Rathod & Di Fulvio, 2021). With the use of less than 20 (arbitrary) intermeal intervals, they did not find broken stick relationships between IMI and the aforementioned meal parameters. In **chapter 5** I showed that by taking into account every IMI reported by the automated weighing system (therefore a greater number than Rathod and Di Fulvio), the relationship between averaged meal sizes and minimal IMIs did show broken-stick relationships.

Importantly, this was done for each animal, as by pooling data from all the mice, broken-stick relationships were not present. This suggests that these relationships can be investigated only when a greater number of IMI is taken into account and with data of individual animals. One shortcoming of such analysis is that it can be time consuming, as meal size has to be calculated several times for each animal. This can be overcome by the use of the R free software, that allows to compute such analyses in an automated and fast way (R Core Team, 2013). In fact, through the use of an R function, average meal size for each given IMI could be calculated for each animal (for a total of 45 mice) in less than a minute. The broken-stick model indicates that at shorter intermeal intervals (relative to the breakpoint) the averaged meal size increases faster. On the other hand, after the breakpoint is reached, averaged meal size increases slower. Therefore, with this model it is assumed that initially average meal size increases faster, as more feeding bouts are clustered into meals (within meals). After the breakpoint is reached, feeding bouts occur less often (between meals) and average meal size increases slower. The advantage of our methodology is that these broken-stick relationships were automatically investigated with the R library "segmented" (Muggeo, 2008). This allowed to overcome one of the biggest biases of previous methodologies used to investigate a meal criterion, as one of the main limitations was that an intermeal interval to separate feeding bouts had to be chosen by visual inspection (Sibly, 1990), making this practice subject to potential bias. To facilitate further research into mouse feeding behaviour (but also for other animals), we are planning to make these R functions freely available online.

Another aspect investigated in **chapter 5** is that the use of standard arbitrary intermeal intervals (i.e. a predefined and arbitrarily chosen criteria for all the animals) to define meals may obscure certain aspects of metabolic and dietary manipulations compared to those obtained with the MCA function for each animal. We came to this conclusion as breakpoints were significantly shorter in mice fed a high fat diet and longer in mice kept at 28°C, indicating that dietary and environmental manipulations affected how meal size develops in relation to increasing IMI, therefore these differences are overlooked with the use of standard arbitrary IMI. This means that if arbitrary intermeal intervals are used to define meals, individual differences on how meal size develops in relation to each given intermeal interval are not taken into account, but the same intermeal interval is used for meal definition, irrespective of diet and temperature. Certainly, the use of arbitrary IMI led to differences in the main effects of diet and temperature, indicating how important it is to take into account and analyse meal related parameters not only using arbitrary IMI. This implies that studies performed with

the use of standard arbitrary IMI may have missed potential relevant effects of experimental and reanalysis of meal related parameters with the use of the MCA function may shed some light in this respect.

In conclusion, in **chapter 5** we showed that (automated) breakpoint analysis is possible and valid on individual data sets of food intake patterns obtained from mice over a 5 day interval and that the use of arbitrary intermeal intervals to define meals should be carefully evaluated. There are also some limitations of our study that deserve to be mentioned. Our automated weighing system could detect only feeding bouts that weighed more than 0.04g, excluding small feeding bouts that may potentially be relevant for food intake analysis in mice. However, this approach allows to rule out behaviours that may be unrelated to feeding behaviour, such as climbing and playing with the food hopper. Future studies should focus on replicating the MCA analysis also with more sensitive systems in mice. The validity of this methodology should be applied also to rats' feeding behaviour. In addition, the MCA system can be used to investigate feeding behaviour and meal related parameters also in socially housed animals, with the help of radio frequency identification (RFID) transponders implanted subcutaneously, as recently done by Rathod and Di Fulvio (Rathod & Di Fulvio, 2021). With this approach, the eating patterns and breakpoints of group housed mice and potential differences among dominance hierarchies can be unravelled.

Conclusions

Overall, in this thesis we showed that social factors during lactation greatly affect growth, energy balance and metabolic health. Furthermore, social factors (individual versus social housing) after weaning, were also able to affect growth, metabolism and body composition, but in a sex-dependent fashion. Therefore, litter composition and postweaning social housing practices have to be thoroughly reported in studies investigating energy balance regulation. In addition, social hierarchy relationships were clear both in male and female socially housed littermates, but these pertained to clear differences in energy balance regulation and metabolic health only in female mice. Finally, investigation of feeding behaviour and meal-related parameters in mice is possible through automated breakpoint analysis and the use of arbitrary intermeal intervals to define meals should be carefully evaluated.

This thesis raises awareness on social, environmental factors and feeding behaviour in a mouse model of obesity research, and how these factors can affect reported outcomes. These findings should be taken into account in future research, since social factors can strongly affect growth, energy balance and metabolic health and feeding behaviour can be affected by the choice of an intermeal interval for meal definition. We recommend being more aware of these specific and potential confounding factors in ongoing preclinical research and when evaluating the efficacy of drugs, nutritional components and other metabolic interventions to enhance scientific (preclinical) progress.

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