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

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Chapter 5

A new method for the assessment of meal parameters in laboratory mice exposed to different experimental and environmental conditions

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Abstract

Mice have been extensively used in feeding behaviour studies. The choice of an intermeal interval (IMI) to define separate meals and as such defining meal related parameters is of importance. Typically, most IMIs are chosen arbitrarily and there are few quantitative methods to assess them in mice. Current methodologies take into account the length and the frequency of intermeal intervals to obtain an IMI. However, as meals are a more relevant unit of animal feeding behaviour instead of the frequency of IMIs, a methodology that investigates the relationship between meal related parameters and IMIs would make more sense in this respect. Therefore, we sought a new method to assess the relationship between meal size and increasing IMIs and investigate whether an IMI could be extrapolated. To do so, feeding behaviour was continuously recorded using an automated weighing scale apparatus for 5 days in 13-weeks-old male C57BL/6J mice (n=45), under specific experimental (low fat vs high fat) and environmental (21°C vs 28°C) conditions. A meal cluster analysis (MCA) function was designed to obtain meal sizes for each given intermeal interval, by iteratively clustering meals. The relationships between meal sizes and minimal IMIs showed broken-stick relationships, in which meal size rapidly increases with increasing IMIs, until a sudden "breakpoint" is reached. Individual breakpoints were automatically extracted for each animal and were used to define meals. The meal related parameters obtained were subsequently compared with meal related parameters obtained using arbitrary IMI. The MCA function successfully revealed breakpoints under each experimental and environmental conditions, as well as under nocturnal and diurnal conditions. Importantly, breakpoint estimations and meal-related parameters were strongly affected by dietary and temperature challenges, indicating that experimental and environmental manipulations affected how meal size progressed in relation to increasing IMI. Differences in the main effects of diet and temperature were dependent on the use of arbitrary IMI (i.e. the same IMI for each mouse, irrespective of experimental and environmental manipulations), indicating that the choice of an arbitrary IMI could influence experimental outcomes. In summary, the current study revealed that breakpoint analysis is feasible and valid on individual data sets of food intake continuously recorded from mice over a 5-day interval, and it also allows to differentiate nocturnal and diurnal phases for breakpoint analysis. Furthermore, using arbitrarily chosen IMIs may obscure certain aspects of metabolic and dietary manipulations compared to those obtained by MCA and their use should be carefully evaluated.

Introduction

Mice have been widely used to unravel the neurobiology of feeding behaviour (Clifton, 2000; Tecott & Abdallah, 2003; Ellacott et Al., 2010). For this purpose, a multitude of genetic modifications, pharmacological tools and behavioural paradigms have been employed in and for mice which advanced the knowledge on regulation of hunger and satiety, taste perception, the role of motivated behaviour on ingestion, and the integral regulation of energy balance (Ellacott et al., 2010; Heisler & Lam, 2017, Tecott & Abdallah, 2003, Lin et al, 2014, Dickson et Al., 2011). However, a persistent problem in the mouse literature is the lack of consistency in the meal-definitions used. Mice, like humans, presumably eat their food interspaced by time intervals of different durations (Strubbe & van Dijk, 2002), and investigators often defined on arbitrary grounds an intermeal-interval (IMI, the time that should minimally elapse between two feeding bouts in order to consider them as separate meals) (Castonguay, Kaiser, & Stern, 1986; Strohmayer & Smith, 1987). The IMIs that are used may therefore vary between studies. For example, IMIs have been reported of 5 minutes (Kim, Park, et al., 2013; Stengel, Wang, Goebel-Stengel, & Taché, 2011), 10 minutes (Dill, Shaw, Cramer, & Sindelar, 2013; Elander, Engström, Hallbeck, & Blomqvist, 2007; Li et al., 2014),15 minutes (Bake, Murphy, Morgan, & Mercer, 2014), 20 minutes (Chi & Powley, 2003; Fox, Biddinger, Jones, McAdams, & Worman, 2013) or 30 minutes (Atalayer & Rowland, 2009). Meal pattern analysis and its primary outcomes - meal frequency and meal size – may rely on such definition, and could therefore impact study outcomes (Castonguay, Kaiser, & Stern, 1986; Strohmayer & Smith, 1987; Demaria-Pesce & Nicolaïdis, 1998). It may be possible that individual differences in feeding behaviour and/or group effects hereon (due to certain treatment/conditions) remain unnoticed or overstated using arbitrary chosen IMIs.

A semi-quantitative method to assess repetitive behaviours (and ultimately IMI) that tend to cluster (like feeding behaviour) is the so-called survivor analysis (Slater & Lester, 1982). In this analysis, log transformed cumulated frequency of the number of behavioural events (like feeding bouts) over time is plotted as a function of the minimal time interval that separates these events (Slater & Lester, 1982; Sibly, Nott, & Fletcher. 1990, Clifton, 2000, Castonguay, Kaiser, & Stern, 1986). This type of analysis generally yields an initial steep negative linear relation, in which the cumulative number of feeding events rapidly declines when the minimal interval between these events increases, until a sudden "breakpoint" is reached (a graphical representation can be found in Supplementary material, figure 2). In the feeding behaviour literature, this breakpoint is the smallest

interval interspacing between clustered feeding events that are then considered "meals". Further increasing the minimal time interval generally yields a less steep negative relation with the log transformed frequency of clustered events, which results from the fact the individual bites added to those meals occurs less frequently as the minimal IMI becomes larger. Such a relation is generally referred to as a "broken stick". Application of this analysis to feeding behaviour indeed reveals such "breakpoints" in a wide variety of species including rats (Clifton, 2000, Castonguay, Kaiser, & Stern, 1986), pigs (Gloaguen et Al., 2013), grasshoppers (Chapman and Beerling 1990) and fowls (Savory, 1986); however thus far have not been shown in mice, at least to our knowledge. The aim of this study was to investigate whether such breakpoints could be assessed in male mice using an automated weighing system for continuous monitoring of food intake. In the likelihood we were able to find these breakpoints using the log survivor analysis, we investigated whether such an approach could also be applied by iteratively clustering "meal size" as a function of increasing minimal IMI (i.e., rather than cumulated frequency as resultant of increasing minimal IMI). By using meal size instead of the cumulated frequency of events happening randomly over time, such a method would have the advantage that the average meal size as well several other meal-related parameters could be directly inferred in relation to the breakpoint and not be related to the number of (cumulated) events. Finally, implications of using arbitrarily chosen IMIs (IMI_{arb}) as meal definitions rather than the minimal IMIs based on breakpoint analysis (IMI_{bp}) were investigated under specific experimental and environmental conditions known to affect energy intake (i.e., low fat diet versus high fat diet feeding, ambient temperature of 21°C versus 28°C, and day versus night). This will shed some light on the implications of a common practice used among researchers, and in particular on whether the choice of an IMI_{arb} can be a moderator of reported experimental outcomes. Finally, by investigating ambient standard temperature versus thermoneutrality, we aim at uncovering how meals are regulated in non-cold temperatures, as studies investigating ambient temperature on meal regulation in rodents are rare and mostly include comparisons between animals kept at standard room temperature versus very low temperatures (Leung & Horowitz, 1976).

Methods

Ethical statement

Experimental procedures were approved by the Dutch Competent Authority ("Centrale Commissie Dierproeven") and all animal experiments and procedures were performed in accordance with the principles of good laboratory animal care following the EU-directive for the protection of animals used for scientific purposes. C57BL/6J mice were chosen as they represent one of the most common inbred mouse strains used in biomedical research. In particular, this mouse strain is prone to obesity and related metabolic alterations and is therefore often used to study feeding behaviours. The animals used for this study were part of a larger experiment to investigate effects of different housing conditions (individual versus social housing) on a variety of metabolic and behavioural outcomes in C57BL/6J mice.

Animals and experimental design

Mice in this study were inhouse bred offspring from naïve breeding couples obtained from Charles River Laboratories (Sulzfeld, Germany). Unless otherwise stated, breeder mice were maintained under conventional conditions in polycarbonate Macrolon type II cages with bedding (wood shaving) and a shelter (red-transparent plastic house), in two separate rooms with a controlled environment (12h/12h light/dark cycle with lights on at 09:00 and off at 21:00; 21 ± 1°C 50 ± 5% humidity), and were provided with grain based control chow diet (LF; Altromin® 1410 – 10 mm pellets; 15.397 KJ/Kg, 22% of total calories from fat, 28% from protein and 50% from carbohydrates) and water *ad libitum*. At postnatal day (PND) 2, litters were randomized and culled to 6 pups (4 males and 2 females or 3 males and 3 females). At weaning (3 weeks), male offspring were single-housed and randomly assigned to be housed at one of two conditions differing in ambient temperature (21 ± 1°C or 28 ± 1°C) and kept on the LF control diet. Single-housing was of the factors investigated in another experiment (chapter 3 of this thesis) and it was required for monitoring of food intake at the individual level, as this would have not been possible in socially housed animals. Ambient temperature of 21°C is considered standard room temperature in most laboratories, while 28°C is considerably higher and closer to mouse thermoneutrality (Speakman & Keijer, 2013; Fischer, Cannon & Nedergaard, 2018). At 6 weeks of age, half of the experimental mice were switched from LF to an obesogenic high fat/high sugar diet (HF; 19.957 KJ/Kg, 44,7% of total calories from fat, 19,6% from proteins and 35,7% from carbohydrates), while the other half of the mice continued on the LF control diet. This resulted in 4 experimental groups: mice kept at 21°C and fed a LF diet (21 – LF; control group, n = 11), mice kept at 21° and fed a HF diet (21 – HF; diet challenge, n=11), mice kept at 28° and fed a LF diet (28 – LF; temperature challenge, n=11), mice kept at 28°C and fed a HF diet (28 – HF; diet and temperature challenge, n=12). All cages were randomly placed in racks in the respective climate rooms with mice having visual, auditory or olfactory contact

with neighboring cages or cages located elsewhere in the room. Data analyses were conducted at the end of the experiment with researchers not aware of group allocations. Diets were provided to the mice on the lid of the cage containing a food hopper, unless mentioned otherwise. The HF diet was prepared in-house by using grounded 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%). Both LF and HF diets were provided to the mice as pellets. The energy content of the experimental diets is represented in table 1. Cages were cleaned one week before the food monitoring during the light phase, where olfactory cues were transferred from the old to the new cages (i.e. a handful of wood shaving). A new lid containing an adapted food hopper was used for the test and was provided at the beginning of the test. Cages were left untouched for the duration of the test. Tap water was provided freshly at the beginning of this procedure. All mice were weighed at the beginning and at the end of the monitoring.

	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

Continuous monitoring of food intake

When mice were 91 days old (PND91), food intake and meal related parameters were monitored continuously during the light and dark phase using an automated weighing scale apparatus (TSE System, Drinking and Feeding monitor) over a period of 6 days of ad libitum food intake. This assessment was carried out in the home-cage of the mice by replacing the wire mesh lid containing the food and drinking bottle by a custom made lid in which a suspended food hopper (Feeding sensor advanced, 259998-SEN/FED) was fitted (next to the drinking bottle) that was connected to the weighing system. The first 24 hours, in which the mice became used to the system and the new food hopper, were excluded from analysis, as an actual reduced food intake was noticed compared to the following days. The registration equipment yielded chronological data sets exported in .xlsx format with changes in hopper weights to the nearest 0.04 gr, over every 10 seconds intervals, and if two feeding bouts had an IMI of less than 50 second, they were clustered beforehand (i.e., to avoid noise by behaviors that would affect hopper weight, such as climbing). Mice were weighed only at the beginning and at the end of the test (PND91 and PND97), to reduce disturbance to a minimum as possible.

Log survivor analysis and meal-cluster analysis (MCA)

Prior to running the R package, analysis of the raw data is essential. The relation between each minimal IMI reported by the automated weighing scale apparatus and the log transformed cumulated frequency of the number of feeding events was inspected both for each mouse or by pooling data across all the mice for each group (log survivor analysis). Clear broken-line relations upon visual inspection were present only when using data for each mouse separately (supplementary material, figure 2), but these were not present when using data pooled from all the mice of a certain group. Afterwards, data of individual mice were investigated using the R package "segmented" (Muggeo, 2008) to investigate whether these relationships fit a broken-stick model (breakpoint). A function was designed to perform log survivor analysis using R-studio. Initially, a General Linear Model (GLM) was fitted to the data using IMI and cumulative frequency as variables. Subsequently, segmentation was fitted to the GLM, with the intermeal intervals to allow piecewise relation of the variable (seg.Z). The validity of the segmented model over a linear model was tested by using the Davies test to test for a significant difference in slope (Davies, 2002). The summary of the segmented model shows the estimated breakpoint, the confidence intervals of the breakpoint and the estimation of the regression lines in continuous data by using iterative computational algorithms (Muggeo, 2008). Because more than one breakpoint can exist (e.g., when multiple meals start to cluster at certain phases of the circadian cycle), the abovementioned analysis probably works best when using a maximal cut-off for minimal IMI. For this purpose, both the log-frequency survivor as well as the meal cluster analysis (see later) was repeated several times in order to use the IMIs that were shorter than either 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 60 minutes as maximal cut-offs. A clear increase of variance in the breakpoint estimation was observed when cut-offs of IMIs longer than 25 minutes were used. This may be the result of the presence of a second breakpoint when longer IMIs are taken into account, therefore the model may not be able to accurately detect only one breakpoint. We considered a cut-off of 20 min. suitable for breakpoint estimation of our dataset (see Supplementary material, Figure 1).

Since meal frequency and meal size are two major determinants of total food intake, we next investigated whether average meal size (in KJ) as a function of minimal IMI also yielded valid broken-line relations (according to the same methodologies as mentioned above). Meal size was obtained by iteratively clustering meals using increasing minimal IMI, by creating an R function (MCA, meal cluster analysis). Briefly, each IMI reported by the automated weighing scale apparatus was used as a hypothetical IMI (IMI_{hyp}) for meal definition. Therefore, the R function allowed for the calculation of the averaged meal size (*total food intake / meal frequency*) for each IMI_{hyp} reported, by clustering feeding bouts separated by IMI shorter than the IMI_{hyp} and by considering separate meals the feeding bouts separated by IMI longer than the IMI_{hyp}. Subsequently, data of individual mice were investigated using the R package segmented, investigating the relationship between the IMI and the relative average meal sizes. Also in this case, clear segmented relationships appeared only when individual mouse data was used, and not when data was pooled from all the mice of a certain group. The breakpoint found by this model was used as the IMI used to analyse meal-related parameters (IMI_{bp}). We also assessed whether these breakpoints could be found for the nocturnal and diurnal phases separately. Once a breakpoint was found for each animal, this was used to separate feeding bouts into meals at the individual level with the use of another function (analysis of meal-related parameters, see later). For a graphical representation of individual breakpoints (both log-survivor analysis and MCA analysis) found with the R package segmented see Supplementary material, Figure 2.

Analysis of meal-related parameters

With the use of another function, meal-related parameters were calculated using the IMI_{bp} found with the meal clustering analysis. Similarly, to the function described above, feeding bouts were clustered together when they were separated by IMI shorter than the IMI_{bp}, whereas they were considered separated meals when feeding bouts were separated by IMI longer than the IMI_{bp}. Once meals (clustered or not) were defined on the basis of IMI_{bp}, average meal size (in KJ), average meal number (24h average number of meals), average IMI interspacing meals and average meal duration were expressed per animal. In the case of clustered meals, the duration of each meal was calculated by summing the duration of each feeding bout added to the IMIs separating the clustered feeding bouts. The satiety ratio (min/kJ) was calculated by dividing each IMI by the amount of food eaten (in kJ) in the preceding meal. Ingestion rate (kJ/min) for each meal was calculated by dividing meal size by meal duration. Total food intake was analysed either in the dark phase only, light phase only or total daily food intake. To compare meal related parameters obtained with the MCA model with arbitrarily chosen IMI (IMI_{arb}), the same function was used with IMIs of 5, 10, 15 and 20 minutes. Total food intake was presented in absolute terms (grams), in terms of energy (KJ) or as proportion of food intake between light and dark phase (%). To investigate the circadian rhythm of food timing, food intake was also investigated per classes of 3 hours over the circadian cycle (09am – 12pm, 12pm – 15 pm, 15pm – 18pm, 18pm – 21pm, 21pm – 00am, 00am – 03am, 03am – 06 am, 06a, – 09am).

Statistics

In order to reduce the number of animals used for scientific research purposes, animals that were used for the current study were part of a larger experiment aimed at investigating effects of different housing conditions on a variety of metabolic and behavioural outcomes in C57BL/6J mice. Sample size for that study was calculated based on previous experience using male C57BL/6J mice. Overall, sample size was calculated with 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 groups (the number of total groups used in the large experiments). An F-test (ANCOVA, fixed effects, main effects and interactions) yielded a minimal group size of 12 animals per group. In the current study data of three subjects had to be excluded from meal-related analysis due to software recording errors. Therefore, group composition was as follows: 21-HF n=11, 21-LF n=11, 28-HF n=12, 28-LF n=11, unless specified otherwise. Each mouse has been considered as a single experimental unit since they were housed individually.

Statistical analyses were performed using R (R Core Team, 2013). All data is presented as mean ± standard deviation (SD) unless otherwise stated, and outcomes were considered significantly different when $p < 0,05$. Two-way ANOVA was used to investigate the effect of diet and temperature on breakpoint estimations, delta body weight (*PND97 - PND91*), meal related parameters (meal frequency, meal size, intermeal interval, meal duration, satiety ratio and ingestion rate; this was done for IMI_{bp} and each IMI_{arb}), food intake (24 hours, dark phase, light phase) and contribution of smallest meals detectable to total meal frequency. Significant interactions were followed by multiple comparisons with Tukey's HSD test (honestly significant differences). To meet the assumptions of parametric analysis, residuals were graphically examined for normal distribution, homoscedasticity and outliers. In addition, variance and normality were confirmed by using Levene's test and Shapiro-Wilk test. Energy (KJ) and (mass) food intake (g) intake calculated over the circadian cycle (24 hours) per class of 3 hours were analysed by a three-way ANOVA with repeated measures (time

x diet x temperature with the identifier as random effect). A linear mixed effect model was used to predict whether the IMI_{bp} and IMI_{arb} (here referred as model), diet and temperature predicted each meal parameter, which were corrected for the repeated measures and with a random intercept for mouse identity. Data are presented as individual data points overlaid on a bar-plot showing mean ± standard deviation (SD) unless otherwise stated.

Results

Effects of temperature, diet, and circadian cycle on food intake.

At the beginning of the test (PND91), mice fed the HF were significantly heavier than mice on a LF diet ($p<0.001$, figure 1A). Delta body weight between PND91 and PND97 presented a significant temperature x diet interaction ($p = 0.02$), suggesting that mice on a HF at 21°C tended to gain less weight compared to mice on a HF at 28°C (figure 2A). However, Tukey post-hoc analysis showed that this difference did not reach statistical significance (21-HF vs 28-HF $p = 0.07$). Mice kept at 21°C showed significantly increased food intake compared to mice kept at 28°C irrespective of diet type, both in the light and dark phase as well as throughout the majority of the circadian cycle ($p < 0.01$, Figure 1C, 1D and table 1). Feeding a HF diet significantly increased food intake in terms of calories (Figure 1C, $p = 0.04$), however, food intake per mass ingested was strongly reduced by HF feeding, particularly at 21 °C (Figure 1D, $p < 0.01$). A three-way ANOVA with time as repeated measures showed an altered diurnal/nocturnal pattern of food intake, with mice on the HF diet having reduced energy intake during the dark phase that was counterbalanced by an increased energy intake over the light phase, compared to LF feeding (Figure 1E, $p < 0.01$). These changes were the result of an increased energy intake of HF-fed mice over the final three hours of the light phase (18 – 21 pm). Conversely, LF-fed mice had a peak of food intake at the onset of the dark phase (21 pm – 00 am) (Figure 1E).

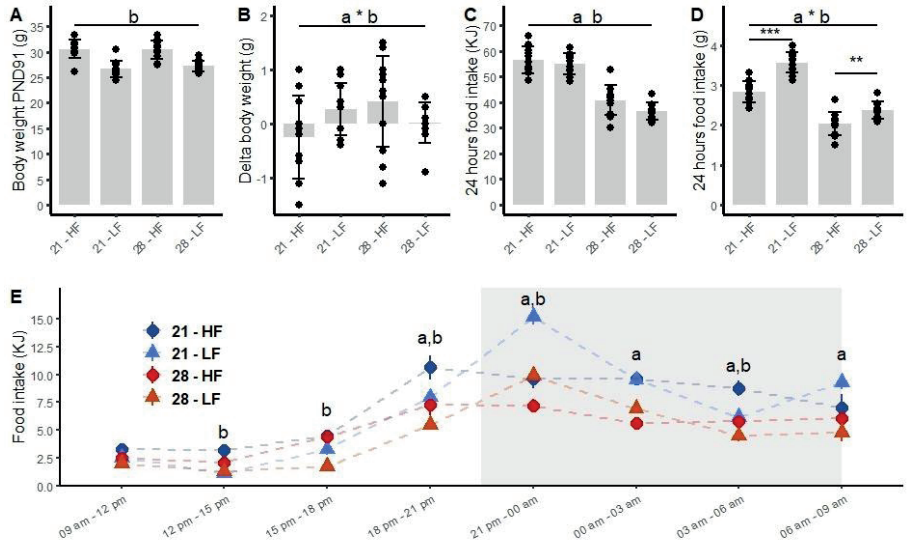

Figure 1. Food intake analysis in male C57BL/6J mice kept either at 21°C or 28°C and fed either a low-fat diet (LF) or a high fat diet (HF). (**A**) Body weight (G) at the beginning of the test (PND 91). (**B**) Delta body weight from the beginning till the end of the test (PND 91-97, grams). (**C**) Averaged 24 hours food intake (KJ). (**D**) Averaged 24 hours food intake (g). (**E**) Diurnal/nocturnal pattern of energy intake (KJ). Data is presented as individual data points and as means ± SD. For graph E, data is presented as means ± SE to allow for better data visualisation. (a= temperature effect, b = diet effect, a*b = temperature * diet interaction).

Group	Food intake (KJ)		Food intake (KJ) %	
A) Dark phase				
21 - HF	35.1 ± 5.2	a,b	62.0% ± 6.6%	b
21 - LF	40.2 ± 3.3	a,b	73.2% ± 5.4%	b
28 - HF	24.6 ± 4.4	a,b	60.2% ± 6.0%	b
28 - LF	26.1 ± 3.9	a,b	71.3% ± 8.0%	b
B) Light phase				
21 - HF	21.5 ± 4.3	a,b	38.0% ± 6.6%	b
21 - LF	14.8 ± 3.5	a,b	26.8% ± 5.4%	b
28 - HF	16.2 ± 3	a,b	39.8% ± 6.0%	b

Table 1. Averaged food intake (KJ) and the proportion of food intake in the light and dark phase, relative to total food intake (%). Data is presented as means ± SD (a= temperature effect, b = diet effect). (a= temperature effect, b = diet effect)

Breakpoint analyses

Assessment of 5-day continuous recording of food intake (to the nearest change of 0.04 gr every 10 sec) allowed us to perform log survivor analysis of cumulated frequency of feeding events as a function of minimal IMI as well as meal clustering analysis as a function of minimal IMI. Both methods revealed significant breakpoints (according to the Davies test) for all mice, with a high level of agreement between the two methods (figure 2A-B). Interestingly, datasets for the meal clustering method did not need to be log-transformed to attain linearity. Breakpoints analysis according to both methods were affected by ambient temperature as well as diet, with mice kept at an ambient temperature of 21°C having breakpoints at shorter minimal IMIs than those kept at 28°C ($p < 0.01$), and mice fed the HF diet having breakpoints at shorter minimal IMIs than mice fed the LF diet ($p < 0.01$) (see figure 2A and 2B). Again, no significant differences could be found between the two methods with respect to timing of breakpoints in these conditions. Both methods also revealed quite comparable breakpoints in the dark and light phase in most of the mice, with again temperature and diet effects in the dark phase as mentioned above ($p < 0.01$ for both), but only a diet effect was observed in the light phase ($p = 0.01$) (only shown for the meal clustering analysis; figures 1C and 1D). Importantly, for the dark phase one statistical unit was removed and for the light phase two statistical units were removed, because no clear breakpoints were visible.

Meal clustering analysis and other meal related parameters.

Because the meal cluster analysis performed equally well compared to the log survivor analysis of cumulated frequency to discover breakpoints, but did not require log transformation, we decided to continue investigating other meal-related parameters as a function of minimal IMIs only using the meal cluster analysis. The outcomes of this analysis were then compared to meal parameters obtained from arbitrary chosen minimal IMIs (IMI_{arb}; i.e., 5, 10, 15 and 20 min). Overall, a linear mixed effect model showed relevant interactions between the model chosen ($IMI_{bp,}$ IMI_{arb} of 5, 10, 15 and 20 min) with both temperature and diet for most meal-related parameters (meal size: model x temperature $p < 0.001$, model x diet $p < 0.001$; meal frequency: model x temperature $p < 0.001$, diet $p < 0.001$, intermeal interval: model x temperature $p = 0.03$, diet $p < 0.001$, meal duration: model x temperature $p < 0.001$, diet $p < 0.001$), but for satiety ratio only main effects were present (model $p < 0.001$, temperature $p < 0.001$ and diet $p = 0.002$) and for ingestion rate a model x temperature interaction ($p = 0.03$) and main effect of diet ($p < 0.001$) were found (figure 3). This indicated that the choice of an IMI affects meal size, meal frequency, intermeal intervals and meal duration, and

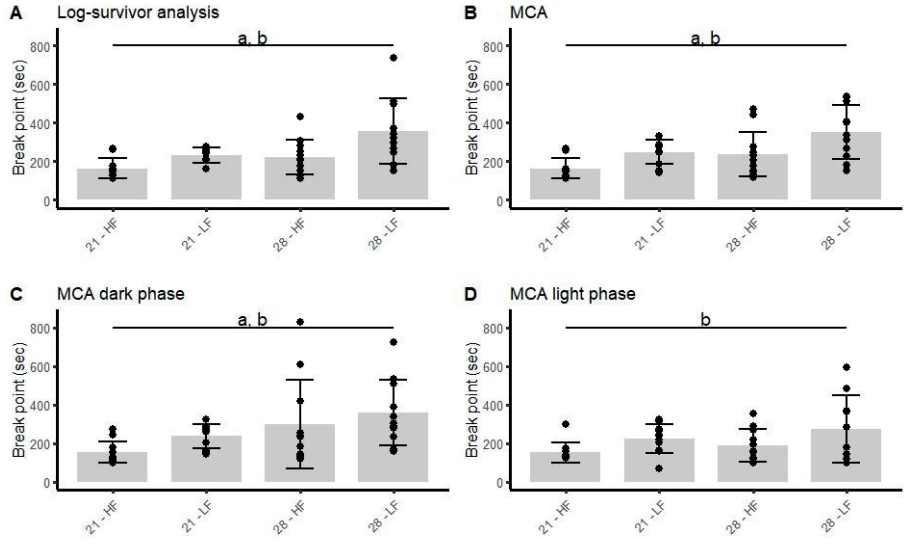

Figure 2. Breakpoint analysis (IMI_{bp}) using the R package "segmented". (**A**) IMI_{bp} obtained through log-survivor analysis and (**B**) IMI_{bp} obtained through MCA taking into account diurnal and light phase combined. (**C**) IMI_{bp} obtained through MCA considering data only from dark phase and (**D**) IMI_{bp} obtained through MCA considering data only from light phase. (21-HF n=11, 21-LF n=11, 28-HF n=12, 28-LF n=11; for C one statistical unit was removed and for D two statistical units were removed). For statistical analysis of C & D data has been log-transformed to achieve normality, but the raw data is presented in the figures. Data is presented as individual data points and as means ± SD. (a= temperature effect, b = diet effect)

the effects that temperature and diet can have on these meal related parameters. Specifically, increasing IMI_{arb} resulted in an increase in average meal size, average IMI, and average meal duration, and lower average frequency and ingestion rate relative to the IMIs at breakpoints (IMI_{bp}). The satiety ratio, however, appeared to be rather constant over the different minimal IMI_{arb} (figure 3E). Interestingly, analyses of meal related parameters for IMI_{bp} and each IMI_{arb} showed that the main effects of diet and temperature were not constant between the models used. In particular, meal size was unaffected by diet with IMI_{arb} of 15 and 20 minutes and was increased by HF feeding with shorter IMI_{arb} (IMI_{bp} $p = 0.01$; IMI_{arb} 5 min $p < 0.001$, IMI_{arb} 10 min $p = 0.01$)(figure 3A). Meal frequency was significantly increased by HF feeding only with an IMI_{arb} of 5 minutes ($p = 0.01$) and unaffected at other IMI_{arb} timings (figure 3B). Average intermeal interval was increased by HF feeding only using an IMI_{arb} of 5 minutes ($p = 0.008$) and unaffected by diet with other IMI_{arb} or using IMI_{bp} (figure 3C). Meal duration was unaffected by temperature when using IMI_{bp} and it was significantly affected with IMI_{arb} of 5, 10, 15 and 20 minutes (IMI_{arb} 5 min $p < 0.001$, IMI_{arb} 10 min $p = 0.001$, IMI_{arb} 15 min $p = 0.002$, IMI_{arb} 20 min $p = 0.003$).

Similarly, ingestion rate was unaffected by temperature when using IMI_{bp} and it was significantly affected with IMI_{arb} of 5, 10, 15 and 20 minutes (IMI_{arb} 5 min p = 0.003, IMI_{arb} 10 min p = 0.007, IMI_{arb} 15 min p = 0.002, IMI_{arb} 20 min p = 0.002).

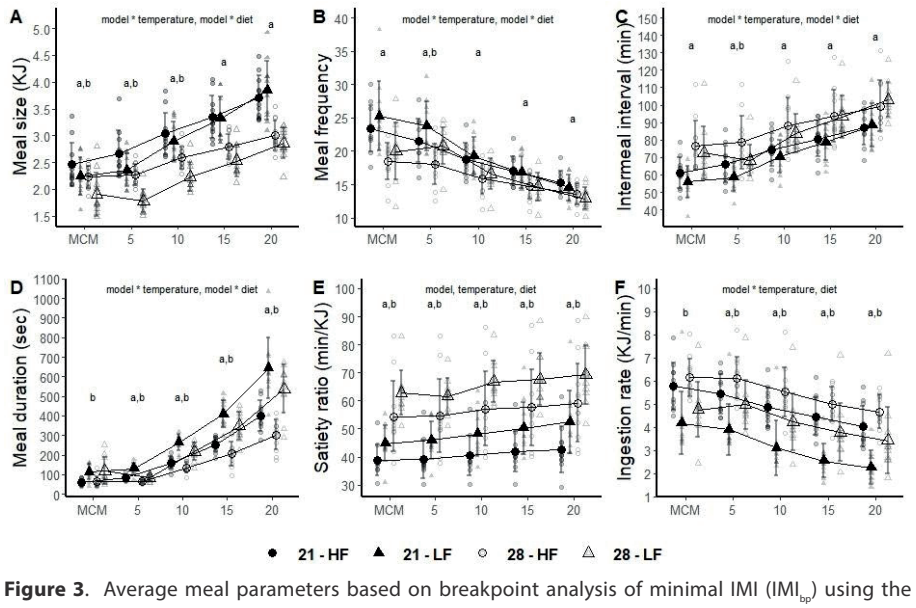

Figure 3. Average meal parameters based on breakpoint analysis of minimal IMI (IMI_{bp}) using the meal cluster analysis (MCA), or based on arbitrary IMIs (IMI_{arb}; 5, 10, 15 and 20 minutes). A linear mixed effect model was used to predict whether the IMI_{bp} and IMI_{arb} (here referred as model), diet and temperature predicted each meal parameter. The result of this analysis is reported on top of each graph (model, temperature and diet effects/interactions are reported). In addition, for each IMI_{arb} of 5, 10, 15 and 20 minutes and the IMI_{bp}, meal-related parameters were statistically analysed (reported with letters: a= temperature effect, b = diet effect). (A) Average meal size (KJ), (B) average meal frequency, (C) average intermeal interval, (D) average meal duration, (E) average satiety ratio (min/KJ) and (F) average ingestion rate (KJ/min) (F). Data is presented as means ± SD. (21-HF n=11, 21-LF n=11, 28-HF n=12, 28-LF n=11). Data has been log-transformed for analysing the meal duration of each model.

Meal clustering analysis and other meal related parameters in the dark and light phase only

Meal clustering analysis was also performed in the dark phase and light phase only, by using the respective IMI_{bp} found for each animal in each phase to cluster meals (reported in figure 2C-D). Interestingly, main effects of diet and temperature differed greatly among the two phases. In particular, meal size was unaffected in the dark phase (figure 4A), but in the light phase it was significantly increased by HF feeding (p < 0.001) and decreased by housing at 28°C (p = 0.02) (figure 5A). Meal frequency was decreased by HF feeding (p = 0.004) and decreased at 28°C (p < 0.001) in the dark phase (figure 4B), but it was only decreased at 28°C in the light phase (p = 0.01) (figure 5B). Average intermeal intervals were longer in HF feeding conditions (p = 0.004) and at 28°C (p < 0.001) in the dark phase (figure 4C); concomitantly they were longer at 28°C (p = 0.01), but unaffected by diet in the light phase (figure 5C). Meal duration was decreased significantly by HF feeding both in the dark phase (p = 0.001,figure 4D) and the light phase (p = 0.03, figure 5D). Satiety ratio was increased at 28°C both in the dark phase (p < 0.001, figure 4E) and the light phase (p < 0.001), but it was only decreased by HF feeding (p < 0.001) in the light phase (figure 5E). Finally, ingestion rate was significantly increased by high fat feeding both in the dark (p < 0.001, figure 4F) and light phase (p < 0.001, figure 5F).

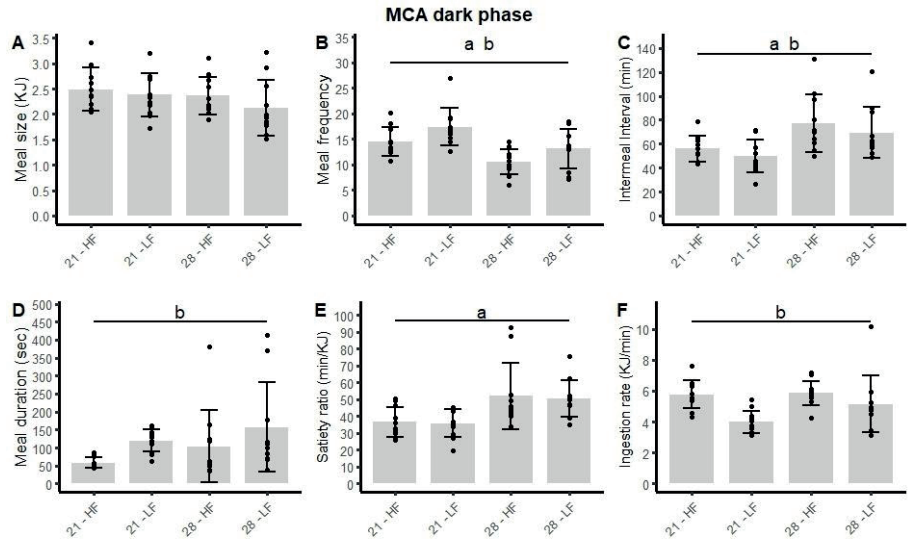

Figure 4. Meal related parameters extracted by the meal cluster analysis using the breakpoints found for each animal in the dark phase. (**A**) Average meal size (KJ), (**B**) average meal frequency, (**C**) average intermeal interval, (**D**) average meal duration, (**E**) average satiety ratio (min/KJ) and (**F**) average ingestion rate (KJ/min). Data is presented as individual statistical units, means ± SD. (21-HF n=11, 21-LF n=11, 28-HF n=11, 28-LF n=11, one statistical unit not used for this analysis as for one animal IMI_{bp} could not be assessed). Data has been log-transformed for analysing the average meal duration, satiety ratio and ingestion rate, but raw data is represented in the figures. a= temperature effect, b = diet effect.

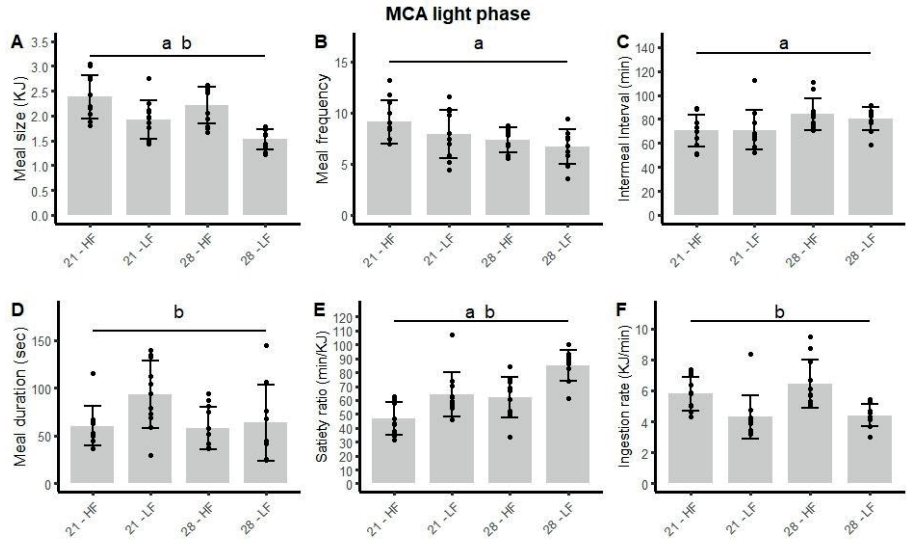

Figure 5. Meal related parameters extracted by the meal cluster analysis using the breakpoints found for each animal in the light phase. (**A**) Average meal size (KJ), (**B**) average meal frequency, (**C**) average intermeal interval, (**D**) average meal duration, (**E**) average satiety ratio (min/KJ) and (**F**) average ingestion rate (KJ/min). Data is presented as individual statistical units, means ± SD. (21-HF n=11, 21-LF n=11, 28-HF n=11, 28-LF n=10, two statistical units not used for this analysis as for two animals IMI_{bp} could not be assessed). One outlier has been removed for meal duration (234 seconds, group = 28-LF n=9). a= temperature effect, b = diet effect.

Contribution of small isolated meals to feeding behaviour

Mice had small isolated feeding bouts relatively frequently. As mentioned earlier, the smallest detected amounts by the weighing system was 0.04g, corresponding to 0.798KJ and 0.616KJ for HF and LF diets respectively. The frequency of both small (i.e., 0.04g) and larger (> 0.04g) meals was lower in mice kept at 28°C compared to 21°C ($p < 0.001$ Figure 6A), however their proportion was unaffected by ambient temperature (figure 6B). Mice on a HF diet consumed fewer larger meals (> 0.04g, $p < 0.001$) and a similar number of small meals compared to LF-fed mice (figure 6A). In agreement with this finding, HF-fed mice had about 46.5% of meal frequency derived from small meals and 53.5% from larger meals, while for LF-fed mice this was 40% and 60% (figure 6B, $p < 0.001$). These results indicate that HF-fed mice, compared to LF-fed mice, had a reduced number of larger meals and a lower proportion of larger to small meals. The contribution of small and larger meals to total food intake was also analyzed. The energy obtained from small meals was decreased at 28°C and increased by HF feeding ($p < 0.001$ for both) and the energy coming from larger meals was only reduced at 28°C ($p < 0.001$) and unaffected by diet (figure 6C). However, the proportion of energy coming from small and larger meals was affected only by diet ($p < 0.001$), specifically, mice on HF had increased energy coming from small meals and less from larger meals compared to mice on LF (figure 6D).

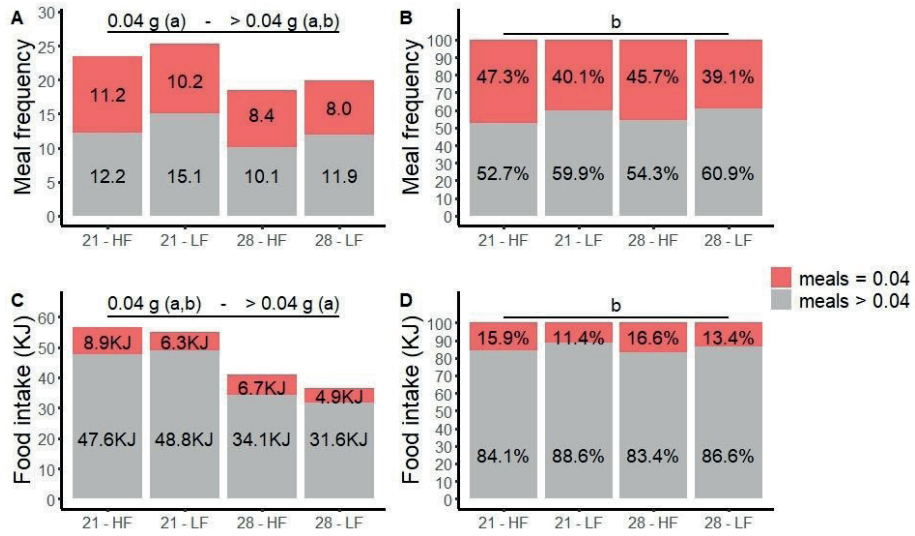

Figure 6. Smallest meals (0.04g) detected by the weighing system and large meals (> 0.04g) and their contribution to meal frequency and total food intake, according to the MCA model. (**A**) Contribution of small and large meals to meal frequency, (**B**) proportion of small and large meals of total meal frequency, (**C**) contribution of small and large meals to total food intake, (**D**) proportion of small and large meals to total food intake. Data are presented as means and averages are reported (21-HF n=11, 21-LF n=11, 28-HF n=12, 28-LF n=11). a= temperature effect, b = diet effect.

Discussion

Our study showed that analyses of feeding behaviour of mice, with changes in food intake to the nearest 0.04 g over every 10 sec, during five consecutive days yields data sets suitable to perform log survivor analysis of cumulative frequency as a function of minimal IMI. For this purpose, we adapted the R package "segmented" (Muggeo, 2008) which yielded breakpoints automatically (thereby ruling out potential bias related to visual inspection) in the majority of mice and under different experimental and environmental conditions. Performance of breakpoint analysis in each individual mouse separately, rather than at the group level, allowed us to perform statistics of differences in breakpoints among the different metabolic and dietary conditions, as well as under nocturnal and diurnal

conditions. Since the principle basis of feeding behaviour in mice appears to be "meal-related" (based on breakpoint analysis) we designed a meal clustering analysis, where the average meal size as a function of minimal IMI directly revealed breakpoints. While the timing of these appeared to be similar to those obtained by log survivor analysis of cumulated meal frequency, the advantage of this meal cluster analysis is that the average meal size as well several other meal-related parameters can be directly inferred in relation to the breakpoint. Interestingly the meal cluster analysis yielded data that, unlike the situation in the log survivor cumulated frequency analysis, did not need to be log transformed, which could be related to the linear nature by which meal size relates to the length of the post-meal interval (LeMagnen & Devos, 1980).

Influence of temperature and diet on energy intake, breakpoints and meal related parameters

The specific environmental condition (ambient temperature) and the dietary interventions used in the current study clearly affected feeding behavior of mice. Specifically, we confirmed that *ad libitum* fed mice kept at 28°C had reduced energy intake compared to mice kept at 21°C and mice on a HF diet presented increased energy intake compared to mice on a LF diet, as previously described by others (Ganeshan & Chawla, 2017; Cui et al., 2016; Licholai et al., 2017). On the basis of the meal cluster analysis, mice kept at 21°C had breakpoints at shorter minimal IMIs than those kept at 28°C, irrespective of whether they were feeding a HF or a LF diet. Thus, the lower energy intake that was observed in mice at 28°C compared to 21°C could be explained by reduced meal size as well as lower meal frequency. As a result, satiety was increased and the rate of ingestion was reduced in mice kept at 28°C. Studies investigating effects of ambient temperature on meal regulation in rodents are very rare and mostly (at least in rats) include comparisons between animals kept at standard room temperature and at very low temperatures (Leung & Horowitz, 1976). Despite differences in body size our findings in mice are however in line with what has been observed in sows (Renaudeau et al., 2002), which suggest meal regulation in relation to ambient temperature may be similarities among endotherms.

Next to ambient temperature affecting breakpoint estimates of average meal size as a function of minimal IMIs irrespective of diet, we found that mice feeding a HF diet had breakpoints at shorter minimal IMIs than those feeding a LF diet, irrespective of ambient temperature. Further analysis revealed that HF feeding mice had higher average meal size, without effects on meal frequency compared to LF feeding mice. It is important to keep in mind that next to a higher energy density, the HF diet was made more palatable with the addition of sucrose, and it had a softer texture than the LF diet. Palatability and texture may affect meal parameters independent of energy/macronutrient content of the diet (Stribiţcaia, Evans, Gibbons, Blundell, & Sarkar, 2020; Mccrickerd & Forde, 2016). The fact that the HF-induced hyperphagia in mice was associated with increased meal size was also shown in studies using rats (Farley et al, 2003; Treesukosol & Moran, 2014). Together with an increased meal size, HF diet caused shorter meal duration, lower satiety and higher ingestion rate, with higher caloric density of the diet probably contributing to this phenomenon. These findings are in accordance with other studies, in which rats feeding HF diets showed specific eating behaviours characterized by larger and faster meals, likely as a result of lower satiety and increased hunger (Furnes, Zhao, & Chen, 2009; Melhorn et al., 2010), although it is difficult to disentangle these effects from the palatability effects. Studies in rats revealed that HF feeding was associated with reduced meal frequency, likely to compensate for the increased meal size. However, in our study, HF-fed mice did not display lower meal frequency compared to LF-fed mice. Differences in meal frequency between studies using HF-fed rats and HF-fed mice could be the result of species differences. Overall, our findings suggest that the hyperphagia in the HF diet group is the result of altered signals which control meal termination, rather than meal initiation (and thus frequency). Changes in satiety (the feeding state of animal after a meal, indicated by the intermeal interval) and satiation (measure of the feeding state of animal during a meal, indicated by meal size and duration) are likely the result of HF feeding (Blundell & Macdiarmid, 1997).

Influence of dark and light phase on breakpoint and meal related parameters

An aspect that we also investigated was to which extent these environmental conditions modulated energy intake distribution and breakpoints during the dark phase and the light phase. This is an important feature of feeding behaviour research, as most studies are carried out either during the dark phase, or during the dark phase together with a portion of the light phase (Kim, Zhang, et al., 2013; Donovan, Paulino, & Raybould, 2007; Zorrilla et al, 2005) rather than over a 24hr period. Energy intake analysis showed that mice on a HF diet had about 39% of their food intake during the light phase, which was significantly higher than the 27% of mice on a LF diet. A possible explanation for this may be that HF feeding disrupts the circadian amplitude rhythms in mice fed high fat diets (Kohsaka et al., 2007). Importantly, mice on a LF diet had increased amplitude of food intake over the circadian cycle, meanwhile the food intake of mice on a HF seemed to be more uniform. The results from this study also suggests that meal analysis using data from a specific time of the day only rather than 24 hours should be interpreted

with caution. This is all the more relevant when assessing meal parameters at breakpoint. Breakpoint analysis confirmed that breakpoints were reduced by high fat feeding in both the light and dark phase, however the effects of temperature on breakpoints were evident only in the dark phase. This resulted in no effects of diet and temperature on meal size in the dark phase, whereas there were effects of both of these conditions on meal size in the light phase.

Arbitrary IMIs affect meal parameters

Because the meal cluster analysis uses the assessment of IMIs at breakpoints at the individual mouse level, meal parameters based on arbitrary chosen IMIs that are the same for all mice could reveal differences in outcomes. Indeed, the use of longer arbitrary IMIs (IMI_{arb}) yielded progressively larger average meal sizes, reduced average meal frequencies, increased average IMIs, increased meal durations and reduced ingestion rates (i.e. the satiety ratios remained rather constant). More importantly, the use of IMI_{arb} also yielded substantial differences in meal parameters between experimental groups, for example between the HF feeding mice relative to the LF feeding mice, where the IMI_{arb} of 5 min versus IMI_{bp} yielded different outcomes on meal size. This indicates that the choice of IMI can influence the outcomes of meal parameter analysis.

Contribution of small meals to meal parameters

The final question we addressed in this study is how small isolated meals contribute to meal parameters. This is an important point since exclusion of small isolated meals is a common practice in feeding behaviour research in mice and rats. Their exclusion is based on the idea that they may be the result of playing behaviour, physical activity on the weighing system not related to ingestion, or to weighing errors (Treesukosol & Moran, 2016). More sensitive apparatuses are likely to detect changes in measurements that are not related to an animal initiating a meal. The lower number of small meals (0.04g) that was observed in mice at 28°C vs that of mice at 21°C mirrored their lower meal frequency at this temperature, suggesting that these can be considered meals rather than errors. The smallest feeding bouts (0.04g) corresponded to ~ 0.8 kJ and ~ 0.6 kJ for HF- and LF-fed mice respectively. While diet type did not affect the number of (small) feeding bouts, HF-fed mice presented increased energy intake from small feeding bouts compared to LF-fed mice when the number of small feeding bouts was normalized per energy intake. These findings suggest that exclusion of small feeding bouts would fail to consider important differences in food intake and feeding behaviour when using diets that differ in energy density. In the current experiment differences between experimental groups were apparent with the use of 0.04 g as minimal value to identify the feeding bout. As (0.04g) could be considered high for mice (1-2 % of total food intake, depending on diet and temperature), differences between experimental groups may become even more pronounced when taking into account also the contribution of smaller feeding bouts that could be detected by more sensitive weighing apparatuses. Thus, it is questionable to exclude small isolated feeding bouts, and their contribution to meal parameters should be properly evaluated.

Conclusions and recommendations for future research

In summary, the current study revealed that breakpoint analysis is possible and valid on individual data sets of food intake patterns obtained from mice over a 5 day interval, and it is even possible to discern nocturnal and diurnal phases for breakpoint analysis. Furthermore, using arbitrarily chosen IMIs may obscure certain aspects of metabolic and dietary manipulations compared to those obtained by IMIbp. While we have made progress in the methodology of meal analysis using an automated cluster algorithm, further research is needed in mice with higher resolution food intake registration equipment, and also the consequences of our findings for several of neurobiological mechanisms that a play a role in satiety may have to be revisited using proper methodologies. The use of log survivor and/or meal cluster analysis for each mouse to be studied is certainly recommended.

Author contributions statement

GK, GvD, LS, SvH designed the research. GK, SvH, WH and JB collected data. GK and NJ analysed the data. GK and NJ wrote the functions and ran the segmented model. GK prepared the figures. GK (LS,GvD) wrote the manuscript. All authors reviewed the manuscript.

Competing interests

The study was funded in part by Danone Nutricia Research. LS is employed by Danone Nutricia Research.

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Supplementary material

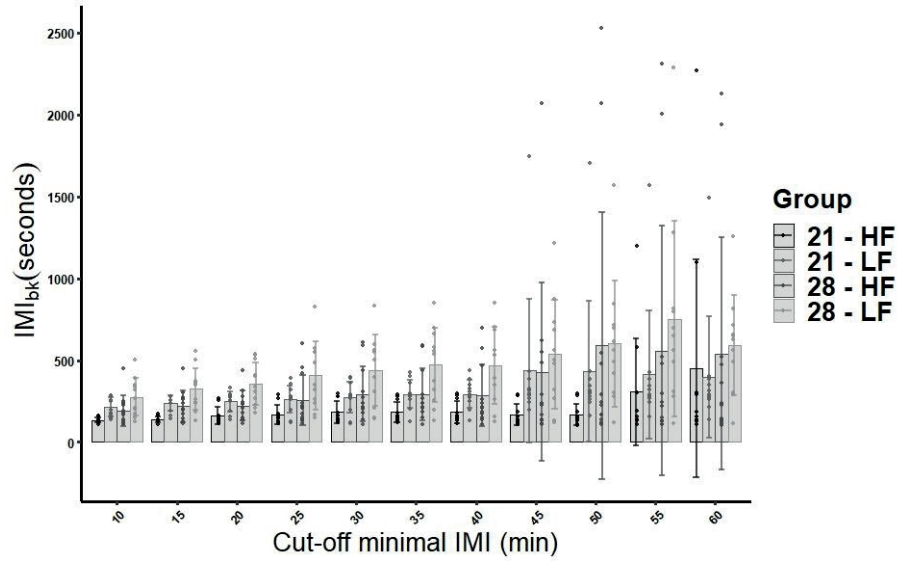

Figure 1. Evaluation of the breakpoint estimations using only intermeal intervals (IMI) shorter than the predefined cut-offs (10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 minutes). The meal cluster analysis was repeated several times with IMIs that were shorter than the predefined cutoffs. The breakpoint found for each predefined cutoff is represented by a dot in the graph and means ± standard deviations are given for each group. This model showed that variation increases when using cut-offs larger than 25 minutes. The choice of an inter-feeding interval cut-off of 20 minutes seems accurate for our dataset, as a clear increase of variance in the breakpoint estimation was observed when cut-offs of IMIs longer than 25 minutes were used

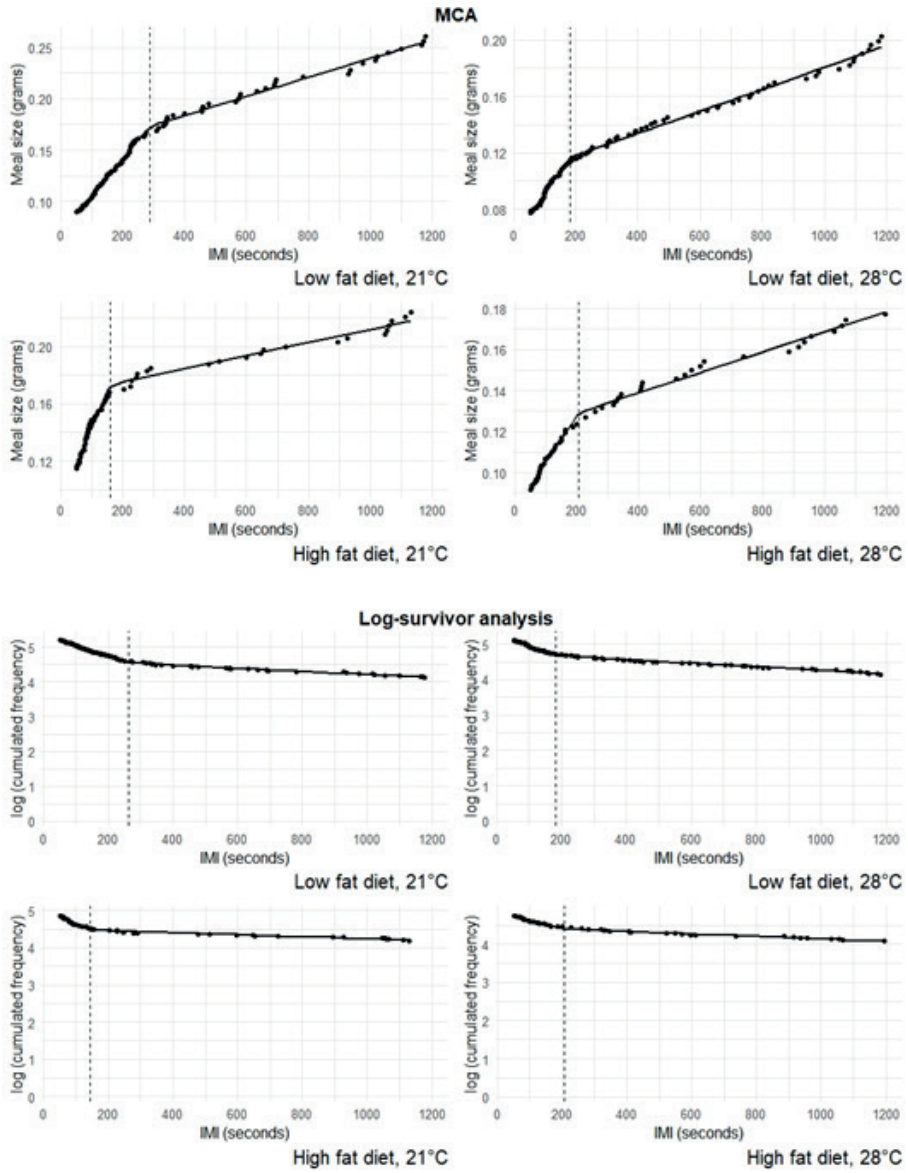

Figure 2. Graphical representation of the segmented model using either the MCA or the log-survivor analysis. One mouse for each group has been represented. The y-axis represents either the averaged meal size in grams (MCA) or the log-cumulated frequency (log-survivor) for each intermeal interval reported by the automated feeding apparatus (x-axis). This way, clear broken-line relationships are visible and dotted lines represent the breakpoints assessed by the R package segmented developed by Vito Muggeo. To perform this analysis, a cut-off on the values of the x-axis has to be established (figure 1) and in our dataset this was fixed at 1200 seconds (20 minutes).