Large, Binge-Type Meals of High Fat Diet Change Feeding Behaviour and Entrain Food Anticipatory Activity in Mice

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Highlights:

- Rapid adaptation of feeding behaviour to scheduled palatable diet access
- No evidence of reduced feeding (hypophagia) prior to scheduled palatable meals
- Mice exhibit food anticipatory activity (FAA) prior to scheduled palatable meals
- Continuing presence of FAA when scheduled palatable meals are withdrawn
- Immediate hyperphagic response once the palatable meals are restored after 7 days

Abstract

Male C57BL6 mice fed *ad libitum* on control diet but allowed access to a palatable high fat diet (HFD) for 2h a day during the mid-dark phase rapidly adapt their feeding behaviour and can consume nearly 80% of their daily caloric intake during this 2h-scheduled feed. To further develop this model, we examined its behavioural characteristics, assessing food intake microstructure and meal pattern, and locomotor activity and rearing as markers of food anticipatory activity (FAA). Analysis of food intake microstructure showed that schedule-fed mice reduced their caloric intake from control diet during the first hours of the dark phase but not during the 3-h-period immediately preceding the scheduled feed. Meal analysis showed that large meal/binge eating behaviour during the 2h-scheduled feed was characterised by increases in both meal number and meal size. Rearing was increased during the 2h-period running up to scheduled feeding while locomotor activity started to increase 1h before, indicating that schedule-fed mice display FAA. Behavioural characteristics such as meal number and physical activity were sustained when HFD was withheld during the anticipated scheduled feeding period, and mice immediately binged when HFD was represented after a week of this 'withdrawal' period. These findings provide important context to the binge-eating model and to our previous studies suggesting that energy balance systems in the hypothalamus are not responsible for driving these large, binge-type meals. Evidence of FAA in HFD dark phase schedule-fed mice implicates anticipatory processes in binge eating that do not involve immediately preceding hypophagia or regulatory homeostatic signalling.

Keywords: feeding pattern, palatability, food anticipation, scheduled feeding, binge eating, mouse

1. Introduction

Feeding is driven, in large part, by energy homeostasis - the balance between food intake and energy expenditure. Humans and many mammals consume their energy in the form of periodic bouts or meals. However, the initiation of a meal is not necessarily based on a general energy deficit or a specific need such as an inadequate glucose level. The impulse to initiate a meal may rather be based on factors such as time of the day, eating habits, social environment, or convenience [1]. The ability to estimate time and anticipate critical events such as meal time is of relevance in nature, since it has clear implications for survival [2]. In laboratory animals, restricted meal-feeding schedules may limit food availability to a single daily meal. Once habituated to these feeding conditions, animals have been shown to anticipate their food through adaptations such as increases in locomotor activity, body temperature and hormone release that precede the predicted meals [3]. The behavioural response is known as food anticipatory activity (FAA), and the 2h to 3h period preceding a daily scheduled meal is the relevant time frame [4-6]. FAA is not just limited to restricted feeding schedules, i.e. where no food is available for most of the day. The reward value of food and its motivational properties have also been implicated in food entrainment since FAA can also be induced in animals fed on palatable feeding schedules, where a stock diet is available for the remainder of the day [7].

A palatable scheduled feeding model, firstly described by Berner et al., [8], based on dietary manipulations by Corwin et al. [9, 10], induces substantial food intake over short periods of time in rats [8]. Utilising this model, we provided scheduled access to a solid high fat palatable diet (HFD) for a 2h-period each day, without imposed caloric restriction during the remainder of the day, a manipulation that resulted in

consumption of large, binge-type meals in both rats and mice [11]. Interestingly, mice exhibited a more exaggerated response to the scheduled palatable diet manipulation, with about 80% of total daily calories consumed during the 2h-access [11]. The present study further characterises the large meal/binge eating model at a behavioural level in mice, focussing on how palatable scheduled feeding influences food intake microstructure and meal patterns. We also measured activity patterns (locomotor activity and rearing) as markers of FAA in mice on scheduled palatable diet. In addition, we extended the model beyond the habituated response to palatable schedule feeding to assess food intake microstructure, meal patterns and activity patterns when the palatable scheduled feeding on HFD was withdrawn and then reintroduced.

2. Materials and Methods

2.1 Animals

6 male C57BL6 mice (Harlan, Bicester, UK), with initial body weights of approximately 22g at 7 weeks of age, were placed under a reversed 12h:12h light/dark cycle (lights on at 16.00, ZT0; lights off at 04.00, ZT12; ZT, zeitgeber time) immediately upon arrival and were allowed to acclimatise as a group. After two weeks, mice were single housed in TSE PhenoMaster/LabMaster feeding/drinking monitoring cages (TSE Systems, Bad Homburg, Germany) and acclimatised for a further week before the start of one week of baseline food intake and locomotor activity measurements (phase 1). All mice were fed *ad libitum* standard pellet diet (Special Diet Services, Witham, UK; #871505 CRM (P); 22% protein, 69% carbohydrate, 9% fat by energy, 2.67 kcal/g) unless otherwise noted. Water was freely available at all times during the experiments. All procedures were licensed

under the Animals (Scientific Procedures) Act of 1986 and received approval from the Ethical Review Committee at the Rowett Institute of Nutrition and Health.

2.2 Dietary manipulation

Following baseline measurements, all mice underwent the same dietary manipulations, performed with pelleted HFD (Research Diets, New Brunswick, NJ, USA, #D12492; 20% protein, 20% carbohydrate, 60% fat by energy, 5.24 kcal/g). During phases 2 and 3, all mice had scheduled access to HFD for 2h a day from ZT18 to ZT20 (6h to 8h into the dark phase) and standard pellet diet in the remaining time (phase 2, adaptation; phase 3, habituation). After 17 days of HFD scheduled feeding, the mice were switched back to standard pellet diet during scheduled feeding time (i.e. standard diet available 24 h a day; phase 4, replacement). After a further 7 days mice were returned to HFD during scheduled feeding time for 7 more days (phase 5, refeeding). Body weight was measured three times a week.

2.3 Food intake measurement and food intake microstructure analysis

During phases 1-5, food intake was measured using the TSE PhenoMaster/LabMaster system, which automatically records the weight of food eaten to a sensitivity of 0.01g through a calibrated sensor. Food spillage was minimised by a catch tray underneath the food hopper. For assessing HFD intake during scheduled feeding, food hoppers containing the diet were exchanged using the ‘food refill’ menu in the software at ZT18 and then again at ZT20. Food hoppers were also exchanged during baseline and replacement phases to standardise the amount of disturbance each day. Cumulative food intake was recorded at intervals of 5 min and summarised in 1 h bins and then averaged per mouse and study phase.

2.4 Meal pattern analysis

Data for meal analysis was collected as binary data every 10 sec. Meal analysis was done as ‘so called’ sequence analysis, whereby all meals occurring during the study period were recorded chronologically to allow the evaluation of single feeding episodes. The start of a meal was defined by food removal equal to or larger than 0.05 g and the meal as ended when no further food removal occurred before the end of the inter-meal interval of 15 min. The meal parameters (meal frequency and meal size) were then summarised over 4 time periods - total day (24h), dark phase, light phase, and scheduled feeding time, and then averaged per mouse and study phase. A 15 min inter-meal interval is commonly used in defining meals in mice [12] and rats [13].

2.5 Locomotor activity measurement and analysis

Activity was measured using a multicage activity monitoring system (Ugo Basile, Comerio, Italy). Each cage had a horizontal sensor frame for monitoring locomotor activity such as walking and running, and a vertical sensor frame for rearing and exploratory activity. Activity was measured as infra-red beam breaks per 15min interval, and was recorded via WinDas 2006 software (Ugo Basile, Comerio, Italy). Horizontal and vertical activity data were separately summarised at 1 h intervals and then averaged per mouse and study phase.

2.6 Statistical analysis

Statistical analysis was performed with SigmaPlot 12.0 (Systat Software, Chicago, IL, USA). Diurnal differences in food intake microstructure and locomotor activity

pattern during baseline were analysed with one-way repeated measures analysis of variance (one-way RM ANOVA). Longitudinal measurements of food intake and physical activity were analysed by two-way RM ANOVA for effect of 'study phase' and 'time point', and interactions between these factors. Data for meal pattern were analysed by one-way ANOVA to reveal overall effects between study phases. Post hoc and planned comparisons were assessed with Student-Newman-Keul Tests (SNK). Outcomes were considered statistically significant if P values were lower than 0.05. Data are presented as mean ± standard error of the mean (SEM).

3. Results

3.1 Food intake and body weight

Study phase had a significant effect on mean caloric intake when analysed in 2h, 22h or 24h bins ($P<0.001$). When mice were schedule fed on HFD for 2h a day, they rapidly adapted their feeding behaviour to scheduled access conditions and binged on HFD, such that by the second day of HFD access, near maximal caloric intake was achieved (Fig. 1A). By contrast, the displacement of calories from standard diet in the remaining 22h occurred more slowly, reaching a nadir after 7 days (Fig. 1B). For this reason the first 7 days are referred to as the adaptation phase. The following 10 days on scheduled HFD were termed the habituation phase since caloric intake during both 2h and 22h bins was relatively stable. The percentages of calories consumed from HFD during the adaptation or habituation phases were 68.3% and 78.0%, respectively, indicative of large meal/bingeing behaviour, compared to just 9.6% of total calories consumed during the same 2h period in the baseline phase. Notably, compensation for calories from scheduled access was incomplete since total caloric intake was increased during adaptation and habituation phases (Fig. 1C)

(SNK, P<0.001 vs. baseline). After 17 days of HFD scheduled feeding, mice were returned to baseline feeding conditions with standard diet during scheduled feeding. 2h caloric intake decreased immediately to a stable lower level whereas 22h intake again adapted more slowly (Fig. 1A, B). Total caloric intake was minimal on the first day of the replacement phase and thereafter increased slowly to baseline levels (Fig. 1C). The overall percentage of calories consumed during the 2h scheduled feeding time was 18.2%, significantly higher than during baseline (SNK, P<0.05). After a further 7 days, mice were again given scheduled access to HFD. 2h caloric intake increased immediately to a level comparable to the habituation phase, and continued to increase gradually across the 7-day phase and was higher on the last day of the refeeding phase compared to several days of the adaptation and habituation phases (day 38; P<0.05 vs. adaptation days 8, 9 11 and 12, and habituation days 15 and 23; P<0.1 vs. adaptation day 10 and habituation days 18 and 24); overall percentage of calories from HFD was at 73.6%. The 22 h caloric intake from standard diet decreased slowly as observed previously in the adaptation phase. Total caloric intake during the refeeding phase was higher than in the adaptation and habituation phases (Fig. 1C) (SNK, P=0.031 and P<0.001).

Body weight reflected changes in caloric intake (one-way RM ANOVA, P<0.001), slowly increasing during adaptation and habituation phases (habituation days 17 to 24, P<0.05 vs. baseline days 1 to 5, and adaptation days 8 to 12), stalling during the replacement phase before increasing again, more rapidly, in the final refeeding phase (Fig. 1D) (refeeding days 34 to 38, P<0.05 vs. all other days; P=0.022 day 36 vs. 34; P=0.055 day 38 vs. 36).

3.2 Food intake microstructure

Analysis of the baseline phase showed that mice displayed a clear diurnal rhythm of food intake (one-way RM ANOVA; $P<0.001$; Fig. 2). Food intake started to increase during the last hour of the light phase at ZT12 (i.e. data from ZT11 to ZT12) (SNK; $P<0.05$ vs. all other ZT intervals) and was always higher during dark phase than during light phase (SNK; at ZT13 to ZT24, $P<0.05$ vs. ZT1 to ZT12). During mid dark phase, food intake was at an intermediate level compared to other dark phase and light phase intervals (SNK; at ZT20, $P<0.05$ vs. all other ZT intervals).

There were significant interactions between study phases and time intervals (two-way RM ANOVA; $P<0.001$; Fig. 2), but no differences between any study phases during the light phase. For clarity, only post-hoc comparisons for effect of study phase within specific 1h ZT intervals are presented below, and Fig. 2 shows comparisons with baseline phase only. During adaptation and habituation phases, schedule-fed mice had increased caloric intake (from HFD) during ZT19 and ZT20 (SNK; $P<0.001$ vs. baseline; Fig. 2E,F). Further, they had a decreased caloric intake from standard diet during the first three hours of the dark phase from ZT13 to ZT15 (SNK; $P=0.043$, $P=0.001$ and $P=0.033$ vs. baseline during adaptation; $P<0.001$, $P<0.001$ and $P=0.003$ vs. baseline during habituation), and a very low caloric intake in the hours following the scheduled feed from ZT21 to ZT24 (SNK; $P<0.001$ vs. baseline; Fig. 2A,B). In contrast, during the 3-hour period running up to the scheduled feed, caloric intake during adaptation or habituation did not differ from baseline (ZT16 to ZT18). During replacement, mice exhibited decreased caloric intake during ZT15, ZT23 and ZT24 dark phase intervals (SNK; $P=0.013$, $P=0.002$ and $P=0.031$ vs. baseline), and retained an increased caloric intake, but from standard diet, during ZT19 and ZT20 (SNK; $P=0.002$ and $P=0.047$ vs. baseline), the scheduled access period (Fig. 2C). During refeeding, food intake pattern on standard

diet resembled the habituation phase with decreased caloric intake during the early dark phase at ZT13 to ZT15 (SNK; P=0.009, P<0.001 and P=0.005 vs. baseline) and late dark phase at ZT21 to ZT24 (SNK; P=0.005, P=0.006, P<0.001 and P<0.001 vs. baseline; Fig. 2D). In addition, mice consumed more calories from HFD during the scheduled feed than in any other phase (SNK; ZT19, P<0.001 vs. all phases; ZT20, P=0.007 vs. habituation and P<0.001 vs. all other phases; Fig. 2G).

Mice consumed 7.85 ± 0.33 kcal HFD within the 2h-scheduled access during the habituation phase. Further analysis in 15-min bins revealed that approximately one third of this intake occurred in the first 15 mins (2.71 ± 0.24 kcal, 34.5% of the 2h-intake, 26.3% of total caloric intake; Fig. 3A). A similar pattern was observed in the refeeding phase, although more calories were consumed in the first 15 mins (3.87 ± 0.17 kcal of a total of 8.81 ± 0.61 kcal, 43.7% of the 2h-intake, 34.2% of total caloric intake; one-way ANOVA, P=0.003 vs. habituation; Fig. 3B). On day one of the refeeding phase, a large proportion of calories were consumed in the first 15 mins (4.62 ± 0.37 kcal of a total of 7.83 ± 0.60 kcal, 59.0% of the 2h-intake, 46.1% of total caloric intake; Fig 3C).

3.3 Meal pattern analysis

There were effects of study phase on both meal number (Fig. 4A) and meal size (Fig. 4B) when analysing over the complete day, the dark phase, the light phase or the scheduled feeding time (one-way ANOVA; P<0.001, P<0.001, P=0.003 and P<0.001 for meal frequency; P<0.001, P<0.001, P=0.001 and P<0.001 for meal size, respectively). Meal number was decreased over the day and the dark phase during adaptation, habituation and refeeding (SNK; day, P=0.002, P<0.001 and P=0.002 vs. baseline; dark phase, P=0.003, P<0.001 and P=0.001 vs. baseline). During the light

phase there were trends towards decreased meal number during adaptation and habituation (SNK; P=0.075 and P=0.057 vs. baseline). However during the scheduled feeding time, mice increased meal number during adaptation, habituation, replacement and refeeding (SNK; P<0.001 vs. baseline). Meal size increased over the day, the dark phase and scheduled feeding time when mice had scheduled access to HFD (SNK; P=0.062, P=0.038 and P<0.001 vs. baseline during adaptation; P=0.023, P=0.016 and P<0.001 vs. baseline during habituation; P=0.003, P<0.001 and P<0.001 vs. baseline during refeeding). Furthermore, meal size during scheduled feeding was largest during the refeeding phase (SNK; P=0.001 and P=0.006 vs. adaptation and habituation). During the light phase, however, meal size decreased when mice had scheduled access to HFD (SNK; P<0.05 vs. baseline during adaptation, habituation and refeeding).

3.4 Activity pattern

Analysis of the baseline phase demonstrated clear diurnal rhythms of both horizontal and vertical activity (one-way RM ANOVA; P<0.001). Horizontal activity started to increase during the last hour of the light phase (SNK; at ZT12, P<0.05 vs. all other ZT intervals) and was elevated during the whole of the dark phase and the first hour of the light phase (SNK; at ZT13 to ZT1, P<0.05 vs. ZT2 to ZT12). There were three peaks in dark phase horizontal activity (SNK; at ZT13, ZT19 and ZT24, P<0.05 vs. remaining ZT intervals), whereas the lowest dark phase levels were observed around the mid dark phase (SNK; at ZT20 and ZT22, P<0.05 vs. all other ZT intervals). Vertical activity at baseline gave a similar picture, with an increase during the last hour of the light phase (SNK; at ZT12, P<0.05 vs. all other ZT intervals), elevated activity during most of the dark phase and in the first hour of the light phase

(SNK; at ZT13 to ZT19, ZT21, ZT23 to ZT1, P<0.005 vs. ZT2 to ZT12), with the exception of ZT20 and ZT22.

There were significant interactions between study phase and time interval for both horizontal activity (two-way RM ANOVA; P<0.001; Fig. 5A-D) and vertical activity (P=0.028; Fig. 5E-H). For horizontal activity, there were no differences between study phases during the light phase. Increases in horizontal activity (vs. baseline) were mainly observed during the mid dark phase: at ZT18 and ZT21 during adaptation (Fig. 5A) (SNK; P=0.022 and P=0.014), at ZT18 during habituation (Fig. 5B) (SNK; P<0.001), at ZT18 and ZT20 during replacement (Fig. 5C) (SNK; P=0.018 and P=0.007), and at ZT18 during refeeding (Fig. 5D) (SNK; P=0.026). Horizontal activity at ZT18 during habituation was higher than all other study phases (SNK; P<0.001 vs. all phases). Decreases in horizontal activity (vs. baseline) were mainly seen during the early dark phase: at ZT13 during habituation (SNK; P=0.008), at ZT13 and ZT15 during replacement (SNK; P=0.011 and P=0.016), and at ZT13 to ZT15 during refeeding (SNK; P<0.001, P=0.046 and P=0.004). In addition there were decreases in horizontal activity at ZT24 during replacement (SNK; P=0.014), and at ZT19 during refeeding (SNK; P<0.001). Horizontal activity at ZT19 during refeeding was lower than the other study phases (SNK; P<0.001 vs. all phases).

For vertical activity, there were similar patterns. Increases in vertical activity (vs. baseline) were mainly observed during the mid dark phase: at ZT17-ZT20 during adaptation (Fig. 5E) (SNK; P=0.007, P=0.001, P=0.002 and P=0.028), at ZT17 and ZT18 during habituation (Fig. 5F) (SNK; P=0.030 and P<0.001), at ZT18-ZT20 during replacement (Fig. 5G) (SNK; P=0.002, P<0.001 and P<0.001) and at ZT18 during refeeding (Fig. 5H) (SNK; P=0.002). The increased vertical activity at ZT18 during habituation and at ZT19 during replacement were higher than all the other

study phases (SNK; at ZT18 during habituation, P<0.001 vs. all phases; at ZT19 during replacement, P=0.027 vs. adaptation and P<0.001 vs. habituation and refeeding). In addition, there was an increase at ZT3 during adaptation (SNK; P=0.023). Decreases in vertical activity (vs. baseline) were mainly seen during the early dark phase. During habituation, there were decreases at ZT13 and ZT15 (SNK; P=0.036 and P<0.001), which were also seen during replacement (SNK; P=0.011 and P<0.001) and refeeding (SNK; P=0.025 and P<0.001). In addition, there was a decrease at ZT24 during replacement (SNK; P=0.031).

4. Discussion

Providing mice with a palatable high fat diet for a 2h-period each day without caloric restriction is very effective in promoting hyperphagia during the access period (Fig. 1A). Consistent with previous reports, when control diet was replaced during scheduled feeding [11], mice rapidly adapted their feeding behaviour and binged on the palatable high fat diet (Fig. 1C), exhibiting a larger binge than rats under the same dietary regime [11, Bake et al., submitted]. To characterise this mouse model at a behavioural level we focused on: (i) differences in the microstructure of feeding behaviour between schedule-feeding (adaptation and habituation phase) and control feeding (baseline phase), (ii) changes in meal size and number under these feeding regimes, (iii) assessment of activity patterns prior to scheduled feeding as a marker of food anticipatory activity (FAA), and (iv) consequences on feeding microstructure, meal patterns and activity patterns when palatable scheduled feeding is withdrawn in favour of control feeding (replacement phase) and then reintroduced again (refeeding phase).

4.1 Changes in feeding microstructure and meal pattern

As anticipated [14], during baseline *ad libitum* feeding conditions, mice showed a clear diurnal rhythm of food intake, consuming most of their food (approximately 85%) during the dark phase (Fig. 2). Food intake was relatively consistent across the dark phase without clear peaks. This is in contrast to the three dark phase peaks observed in rats during early- , mid- and late-dark phase, the latter of which is the highest [Bake et al., submitted].

When mice were schedule-fed on palatable HFD there was a shift in food intake towards the mid-dark phase. Schedule-fed mice showed a large reduction in control diet intake during the early hours of the dark phase, and in the hours following the scheduled feed. However, notably, and in line with our previous observations in rats [Bake et al., submitted], schedule-fed mice did not reduce their control diet intake during the 3h period running up to scheduled feeding on HFD (Fig. 2A,B), the time frame for food anticipation and FAA. These observations suggest that schedule-fed mice were not in a hypocaloric, negative energy balance, state immediately prior to schedule feeding.

Analysis of food intake microstructure in 15-min bins indicated that schedule-fed mice consumed HFD across the 2-h access period (Fig. 3A). The first 15-min bin saw the highest intake, yet only accounted for approximately one third of HFD intake during the access period. The same analysis in rats suggested that a state approaching satiety was reached after 15 min of access since schedule-fed rats consumed approximately three quarters of their intake of HFD in this time [Bake et al., submitted]. Species differences in postprandial satiety with schedule-fed palatable diets may be an interesting avenue for further investigation.

Hyperphagia during scheduled feeding time was due to mice eating more frequent (Fig. 4A) and substantially larger meals (Fig. 4B). The increase in meal size during scheduled access resulted in meals that were 3.4 and 3.6 times higher during adaptation and habituation phases compared to baseline phase. Larger meal sizes have been reported previously in rats fed *ad libitum* on high fat pellet diet [15] or high fat liquid diet [16], as well as in rats prone to DIO compared to DIO-resistant rats fed on a high fat diet [13].

4.2 Entrainment of FAA

Despite the limited effect of scheduled feeding on food intake microstructure during the 3h period prior to HFD access in both mice (current study) and rats [Bake et al., submitted], there were substantial changes in activity pattern in this time frame. The 2h to 3h period preceding a daily scheduled meal is regarded as the crucial time frame for FAA [4-6]. Anticipation of 'mealtime' can be observed in a range of behaviours, including wheel running, lever pressing, activity directed at feeders, general cage activity, and drinking, and represents a laboratory analogue of natural foraging behaviours [4, 7]. In the current study, we utilised general cage activity in the form of locomotor activity (horizontal activity) and rearing (vertical activity) to address whether FAA could be observed as a habituated feeding response in a palatable scheduled feeding regime with HFD. The diurnal rhythm seen in food intake during baseline was reflected in the pattern of both locomotor activity (Fig. 5A-D) and rearing activity (Fig. E-H), with peaks at the beginning, middle and end of the dark phase, a predictable nocturnal pattern [14]. However, it is important to note that whereas food consumption was recorded automatically, diet changes for scheduled feeding were done manually, in the absence of automated access hardware.

Consequently, activity peaks during the mid-dark phase (at ZT19 and ZT21) will have been influenced by the need to manually change the food hopper. To control for this disturbance effect, the physical manipulations were performed daily throughout all study phases, even when no actual change of diet was required during baseline and replacement phases.

The diurnal pattern of activity seen during baseline persisted when mice were schedule-fed on HFD, albeit with a lower intensity during the early hours of the dark phase. There was no major shift in activity towards different time points. Crucially, rearing activity was increased in the 2h period prior to scheduled feeding once the feeding behaviour was habituated to scheduled access conditions, and locomotor activity was increased during the 1h-period prior to scheduled feeding. This increase in activity during that 2h period prior to palatable scheduled feeding is strongly indicative of FAA. Most studies investigating food anticipatory behaviour/FAA have employed restricted feeding schedules, which induce robust increases in activity in anticipation of the predicted meal, i.e. when food is not available. In rats, increases in locomotor activity are observed 2-3h prior to meals of chow in the light phase [17-19]. FAA has been shown in mice prior to daily meals of chow in the mid-light phase through an increase in the combined activity rate for walking, hanging, jumping and rearing during the 3h period prior to a 2h meal [20], as an increase in wheel running during the 3h period prior to a 4h meal [21], or as an increase in locomotor activity during the 2h period prior to a 4h meal [22]. Mice may be capable of anticipating 2 or 3 meals per day [23]. However, fewer studies have investigated food anticipatory behaviour under palatable feeding schedules similar to the one used in the current study. In rats, FAA was observed on access to palatable food in the mid-light phase [24, 25]. However, in some studies, FAA occurred with a lower intensity [18] or not in

all animals of the study population [17]. In mice, it has been shown that FAA has some diet specificity; a palatable feeding schedule with high fat diet [26], peanut butter or cheese [27] induced a moderate increase in high intensity activity (walking, hanging, jumping and rearing) during the 2h period prior to mealtime in the late light phase, whereas mice on a palatable feeding schedule with chocolate or Fruit Crunchies (nutritionally balanced fruit-flavoured pellets) did not exhibit FAA [26].

In most studies investigating FAA, the food is given in the light phase to make observation easier, although one study of mice investigated FAA prior to feeding for 2h at the beginning of the dark phase [28]. However, light-phase manipulations will disrupt the sleep-wake cycle [29]. For example, feeding or forced activity for 8h during the normal resting phase desynchronises the rhythm between the suprachiasmatic nucleus (SCN), the so-called the light-entrainable oscillator, and the liver, disturbs molecular rhythms within the liver, and leads to a loss of blood glucose rhythm and to overweight in rats [30, 31]. Similar consequences have been shown for mice sleep restricted for 6h during the light phase or fed during the light phase only, with rhythms of metabolic genes or circadian genes in the liver being disturbed [32, 33]. Moreover, feeding mice a high fat diet during the light phase was reported to contribute to weight gain in comparison to high fat diet feeding in the dark phase only [34]. In the current study, we show that it is possible to characterise FAA superimposed on normal activity during the active dark phase when mice are not food restricted.

The entrainment of FAA and the timing of meals have been linked to the so-called food entrainable oscillator (FEO) that can work independently of the circadian clock in the SCN [2, 35], although it requires a predictable gap between food presentations, an entrained circadian periodicity of 23-29h, and will persist for

several cycles despite continuous fasting indicates the presence of an independent food clock [5, 36]. Many studies have attempted to locate the FEO in the central nervous system, but its whereabouts have still to be determined, and the molecular mechanisms mediating food anticipation are not fully understood [5, 7]. The FEO might be a distributed network of interacting nuclei each with a different function in the process of mediating FAA, rather than a single structure [7, 35].

4.3 Consequences of withdrawing and reintroducing palatable meals

Previous exposure to the palatable scheduled feeding regime had consequences for body weight, food intake microstructure and meal pattern, as well as activity pattern in the later study phases. After the initial increase during adaptation, body weight plateaued during habituation. However, body weight decreased after withdrawal of HFD during the replacement phase, and then rapidly increased after reintroduction of HFD. It is reasonable to assume that further cycles of replacement and refeeding would lead to pattern of weight cycling, as seen in studies when mice were subjected to two or three consecutive cycles of alternating high fat diet and chow [37, 38]. It has also been shown that weight cycling under such conditions leads to substantial modification of blood lipids, glucose and insulin homeostasis, adipokine levels, and proinflammatory cytokines [37], and a structural remodelling of the liver [38], changes which were not reversed when mice lost body weight during the switch to chow feeding [37, 38]. In addition, the increase in adiposity resulting from high fat diet feeding cannot easily be reversed by reducing body weight when switching back to chow feeding; mice retained the increased number of adipocytes that were accumulated during high fat diet feeding, although adipocyte volumes were reduced [39]. A decreased activity level had been suggested as a responsible mechanism for

weight gain during weight cycling [37]. However since the overall activity rate is not decreased during refeeding (data not shown), the increased body weight in the current study is likely due to the higher total daily caloric intake.

Both feeding microstructure and meal pattern showed that previous experience with scheduled access to HFD had consequences for behaviour during replacement and refeeding stages. When schedule-fed mice were switched back to control feeding conditions during the replacement phase, they retained a meal number appropriate to the consumption of larger amounts of food during that 2h-schedule-fed period (Fig. 4A), had increased caloric intake during that period (Fig. 2C), but returned to baseline meal size. When mice were then returned to HFD during scheduled feeding in the refeeding phase, they had an elevated caloric intake during scheduled feeding compared to habituation. Firstly, this was due to an increased meal size (Fig. 4B), which was 4.4 times higher during refeeding than during baseline and also higher than during habituation. Secondly, intake in the first 15 min following presentation of HFD was higher than during habituation (Fig. 3B). In particular, on day 1 of the refeeding phase, there was an elevated caloric intake during the first 15 min (Fig. 3C). It appears likely that the mice were still anticipating HFD since they continued to exhibit FAA prior to and during the scheduled feeding time throughout the replacement phase.

Consistent with the persistent increase in activity during the replacement phase, it has been shown previously that FAA can persist following withdrawal of palatable diet from a palatable scheduled feeding regime. In rats, for example, FAA persisted under *ad libitum* feeding conditions for at least 7 days at the expected time of a chocolate snack [40], and for mice an increased food bin entry and high intensity activity continued after withdrawal of a palatable high fat treat [26]. In contrast, FAA

disappeared when a period of *ad libitum* feeding followed a restricted feeding schedule when chow was only available for 2h or 3h in the light phase, but FAA was reinstated when rats were fasted [4, 40]. Mice, however, continued to exhibit limited FAA under *ad libitum* feeding conditions following the interruption of a restricted scheduled feeding regime of daily 4h-access to chow in the light phase [21]. The FAA during habituation as well as the persistent FAA during replacement indicate that FAA might be driven by a FEO with a periodicity of 24h but which does not depend on signals of either hunger or nutritional origin.

4.4 Conclusions and possible mechanistic underpinning of binge eating

Scheduled feeding on HFD stimulates a substantial binge eating episode in this mouse model. However, the period immediately before scheduled feeding is characterised by near normal levels of caloric intake from stock diet. We have previously observed the same phenomenon in schedule-fed rats [Bake et al., submitted]. The absence of relative negative energy balance, in advance of the initiation of the binge, in either species, is in line with our previous findings [11], where there was no evidence of potentially causative perturbation in expression of hypothalamic homeostatic neuropeptide genes, such as neuropeptide Y (NPY) or cocaine- and amphetamine-regulated transcript, prior to consumption of large binge-type meals. Similarly, analysis of the gut hormones, ghrelin and glucagon-like peptide-1, indicated that these hormones were not involved in the anticipation of large palatable meals in rats [Bake et al., submitted], whereas they have been implicated in the anticipation of daily meals on restricted feeding schedules [24, 25, 41, 42]. Two key findings of the current study were the continuing presence of FAA and sustained increase in meal frequency during the replacement phase, when only

stock diet was available, and the immediate hyperphagic response once HFD was restored after 7 days. The presence of FAA suggests that this could be part of the priming process for binge eating in the palatable schedule-fed model, which can be initiated very rapidly once HFD becomes available again. Although the mechanistic basis of FAA and the neuronal structures involved have not been definitively established, examination of mouse lines suggests that this behavioural profile is not dependent upon individual hormones or neuropeptides such as leptin [20, 43], ghrelin, NPY or orexin [20], or the histaminergic system [28], although ghrelin receptor signalling might be at least necessary to augment FAA [21, 22], but may require functional dopaminergic [28], serotonergic [44] or melanocortin-3 receptor dependent signalling systems [45, 46]. This suggests an association between the mechanisms underlying binge eating on a palatable diet and FAA, and highlights the value of palatable scheduled feeding models for further investigation as we seek to gain additional insight into the control of meal feeding and over-consumption of calories.

Acknowledgements

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Figure legends

Fig. 1. Caloric intake (kcal) and body weight (g) of C57BL6 mice during all study phases, i.e. mice have either 2h-scheduled access to a high fat diet (HFD) and standard diet in the remaining time (adaptation, habituation, refeeding) or 24h-access to standard diet (baseline, replacement). (A) Caloric intake from either HFD or standard diet during the 2h scheduled feeding time. (B) Caloric intake from standard diet during the remaining 22h. (C) Total daily caloric intake. (D) Body weight. Percentages above dataline in A refer to calories consumed from HFD or control diet during schedule feeding time relative to total 24h intake. Open circles, phase1, baseline; light grey circles, phase 2, adaptation; dark grey circles, phase 3, habituation; grey squares, phase 4, replacement; black squares, phase 5, refeeding. Data are presented as mean ± SEM.

Fig. 2. Food intake microstructure of C57BL6 mice during all study phases vs. baseline food intake pattern: (A,E) adaptation, (B,F) habituation, (C) replacement and (D,G) refeeding phase. (A-D) Food intake microstructure showing caloric intake from standard diet. (E-G) Food intake microstructure showing total caloric intake including calories from HFD during scheduled feeding. Light shaded area indicates dark phase; dark shaded area indicates scheduled feeding time. *P<0.05 vs. baseline by two-way repeated measures ANOVA and Student-Newman-Keul post hoc test. For clarity, one asterisk also includes P<0.01 and P<0.001, and diagrams (E-G) display only differences during scheduled feeding time vs. baseline (ZT19 and ZT20). Data are presented as mean ± SEM.

Fig. 3. Microstructure of caloric intake from HFD during the 2h scheduled access during (A) habituation phase, (B) refeeding phase and (C) day 1 of the refeeding phase. Data are shown as absolute values in 15-min bins. Percentages above bars refer to calories consumed in the 15 min bins relative to 2h intake during scheduled feeding time. Data are presented as mean ± SEM.

Fig. 4. Meal analysis of C57BL6 mice during all study phases. (A) Daily meal number and (B) meal size, presented as daily total, and broken down into dark phase, light phase and scheduled feeding time. *P<0.05 vs. baseline by one-way ANOVA and Student-Newman-Keul post hoc test. For clarity, one asterisk also includes P<0.01 and P<0.001. Data are presented as mean ± SEM.

Fig. 5. Activity pattern of C57BL6 mice during all study phases vs. baseline activity pattern: (A,E) adaptation, (B,F) habituation, (C,G) replacement and (D,H) refeeding phase. (A-D) Locomotor activity pattern (horizontal activity). (E-H) Rearing pattern (vertical activity). Light shaded area indicates dark phase; dark shaded area indicates scheduled feeding time. *P<0.05 vs. baseline by two-way repeated measures ANOVA and Student-Newman-Keul post hoc test. For clarity, one asterisk also includes P<0.01 and P<0.001. Data are presented as mean ± SEM.

Figures

Fig. 1

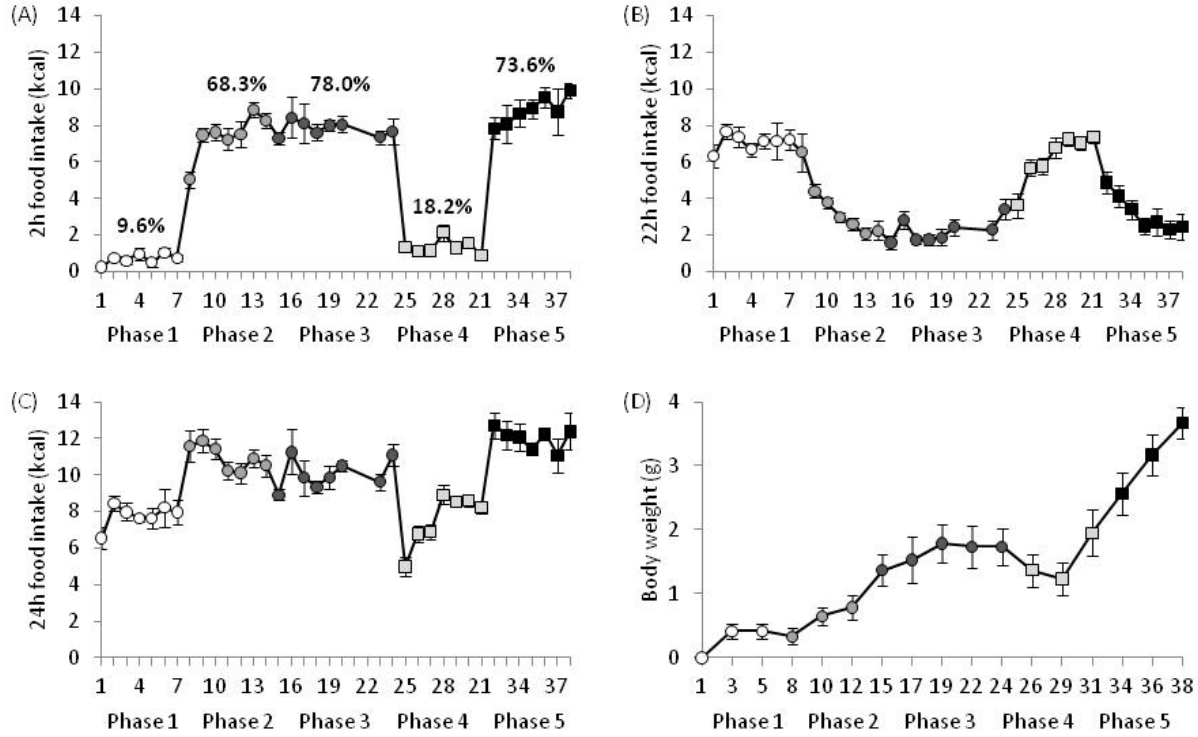


Fig. 2

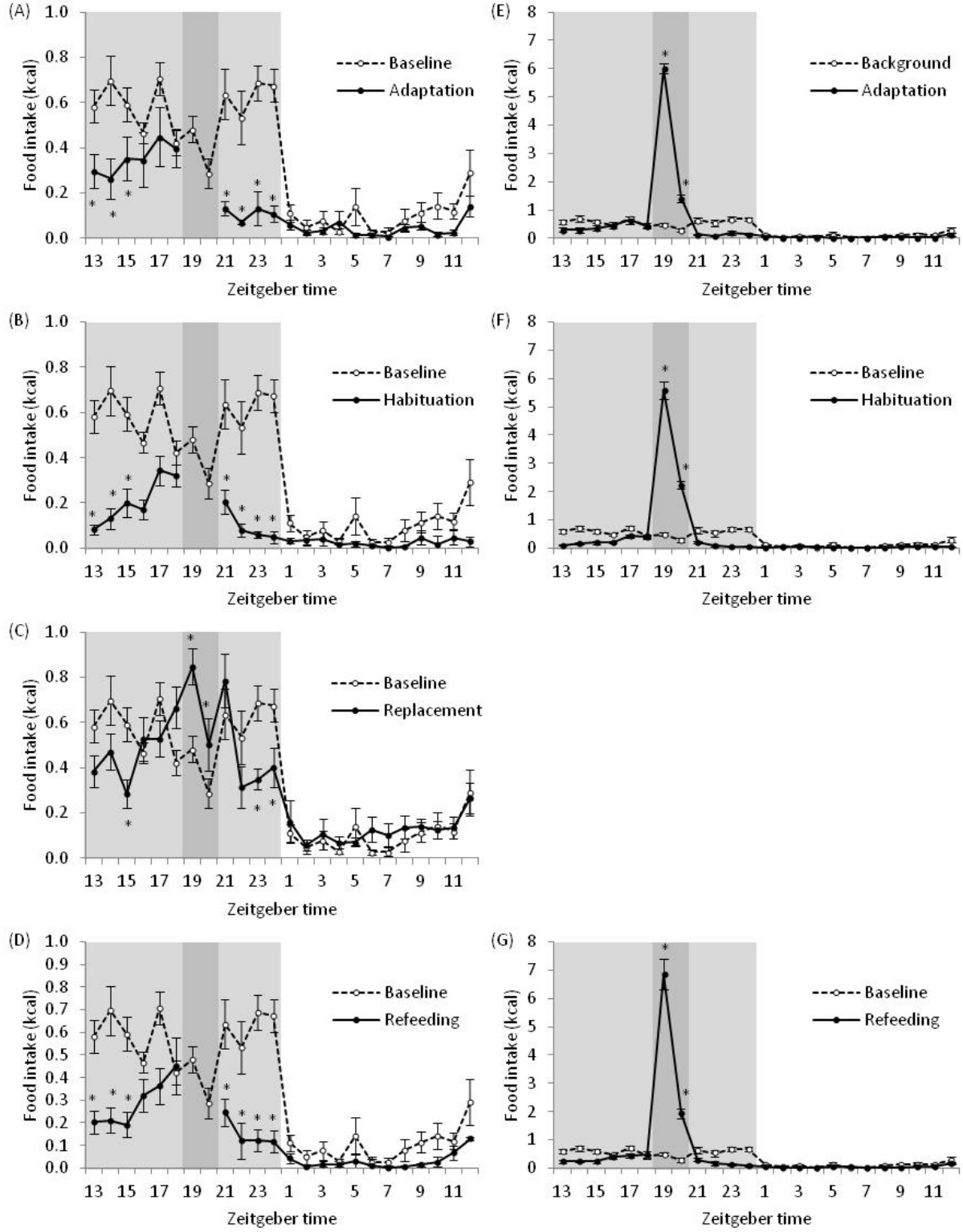

(A)
Food intake (kcal)
Baseline
Adaptation
Zeitgeber time
(B)
Baseline
Habituation
(C)
Baseline
Replacement
(D)
Baseline
Refeeding
(E)
Background
Adaptation
(F)
Baseline
Habituation
(G)
Baseline
Refeeding

Fig. 3

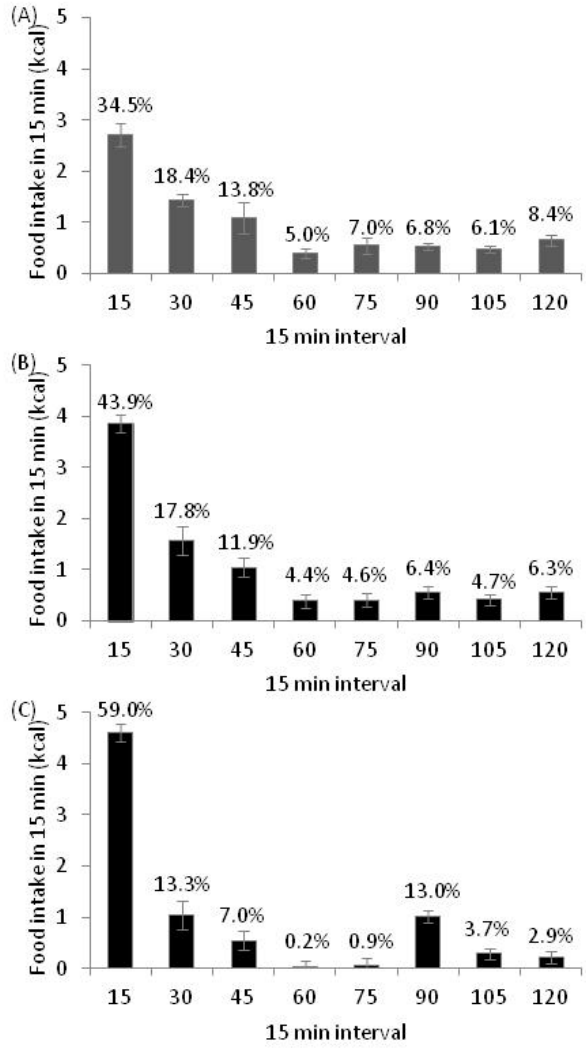

(A)
Food intake in 15 min (kcal)
34.5%
18.4%
13.8%
5.0%
7.0%
6.8%
6.1%
8.4%
15 30 45 60 75 90 105 120
15 min interval
(B)
Food intake in 15 min (kcal)
43.9%
17.8%
11.9%
4.4%
4.6%
6.4%
4.7%
6.3%
15 30 45 60 75 90 105 120
15 min interval
(C)
Food intake in 15 min (kcal)
59.0%
13.3%
7.0%
0.2%
0.9%
13.0%
3.7%
2.9%
15 30 45 60 75 90 105 120
15 min interval

Fig. 4

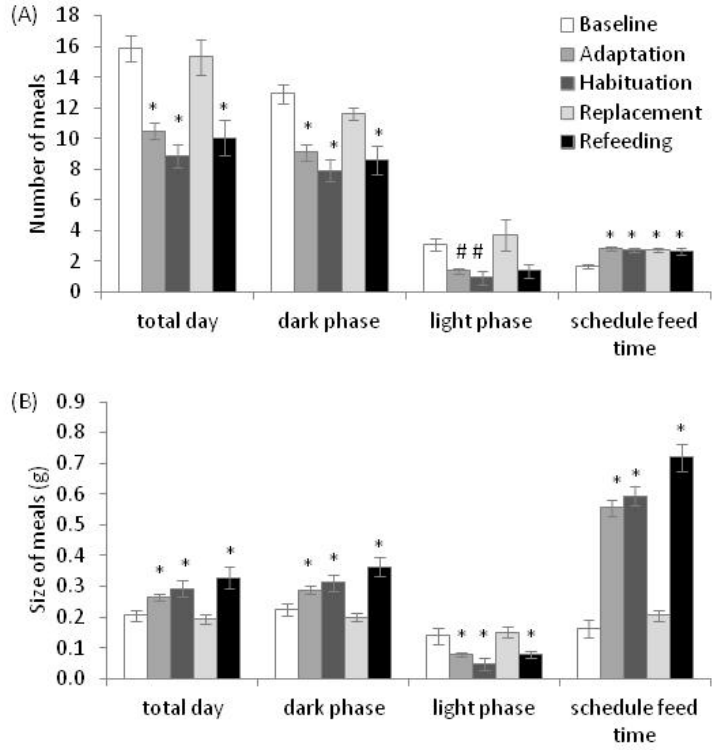
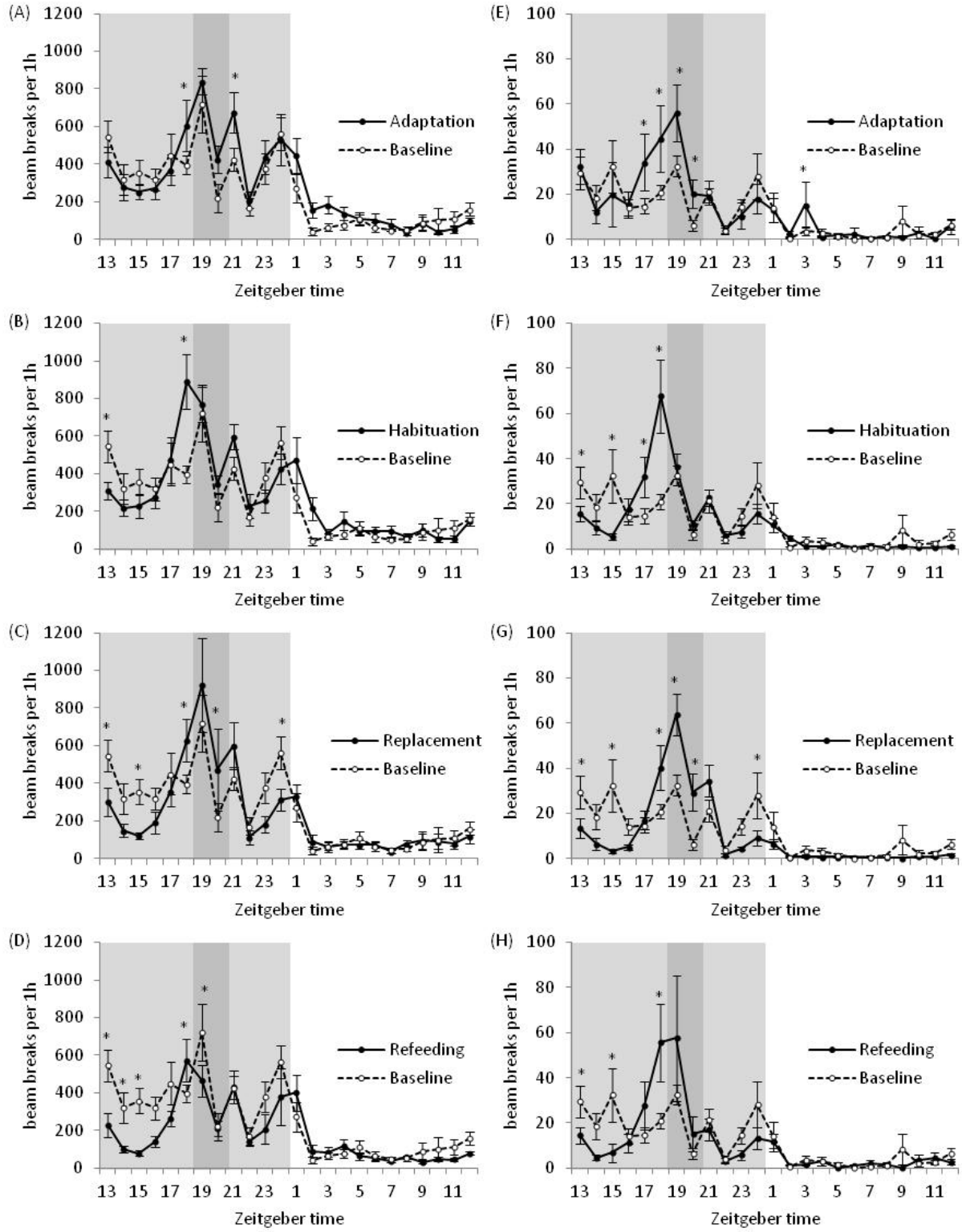

(A)
Number of meals
Baseline
Adaptation
Habituation
Replacement
Refeeding
total day
dark phase
light phase
schedule feed time
(B)
Size of meals (g)
total day
dark phase
light phase
schedule feed time

Fig. 5


(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
beam breaks per 1h
Zeitgeber time
Adaptation
Habituation
Replacement
Refeeding
Baseline