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Post-restriction hyperphagia and metabolic responses to short-term calorie restriction in C57BL/6 mice

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ABSTRACT

Aims: To evaluate post-restriction hyperphagia (PRH) responses to short-term calorie restriction (CR) and the potential drivers of this behaviour.

Methods: Adult male C57BL/6J mice underwent 30% CR for 5 to 30 days, then refed for 12 days. Energy intake, body mass, fat mass, fat-free mass, body temperature, and physical activity were measured continuously throughout the CR and re-feeding phases and daily energy expenditure was measured over final 2 days of CR and the first 5 days of re-feeding.

Results: Following restriction, energy intake, body mass, fat free mass, body temperature and daily energy expenditure were reduced in all groups compared to controls ($P<0.05$). Only the 20d and 25d groups had significantly lower fat mass than controls ($P=0.004$). Total physical activity and dark-phase physical activity did not differ between control and CR groups ($P=0.446$ and 0.380 respectively); but light-phase physical activity of groups 20d, 25d and 30d increased significantly ($P<0.001$) due to food anticipatory activity. All CR groups displayed peak PRH on day1 of refeeding. Total energy intake over the following 2-5 days of refeeding was also greater than the controls ($P=0.002$). The magnitude of PRH increased with CR duration and body mass loss at the individual level ($P<0.001$). In a multiple regression analysis fat free mass loss was the main factor that was correlated with the level of PRH (Multiple regression $R^2=32.7\%$, fat mass $P=0.036$, fat free mass $P=0.003$).

Conclusion: Hunger (reflected by PRH) was mostly related to body mass and fat free mass loss. The effect of fat free mass loss was the opposite of that expected if fat free mass is a key driver of food intake as recently postulated. Developing restriction protocols that minimize loss of fat free mass may reduce the level of hunger that emerges when individuals are under restriction.

Keywords: Energy Intake, Calorie Restriction, Fat mass, Fat-free mass, Post-restriction Hyperphagia

Introduction

Calorie restriction (CR) remains one of the few non-genetic manipulations that results in a robust increase in both life and health span[1-4]. Widely studied in rodents it has positive impacts on lifespan in a wide range, but not all, organisms[2,5]. Greater levels of restriction generally lead to greater increases in average and maximal lifespan up to at least 65% restriction[6-8]. However, among inbred strains of rodents and fruit flies the impacts on lifespan vary enormously, including life shortening in some strains[9-11]. These data suggest that the impacts of CR interact with a large genetic component. Hence, while CR may be a powerful, yet simple, dietary manipulation, potentially applicable in humans[12-14], the individuals with the genetic make-up that will interact with CR to provide the maximal beneficial outcomes remains unclear.

When on restriction rodents experience increased hunger[15-17]. Hence, another problem that is faced by the application of CR to humans is that, unlike rodents placed under restriction that have no choice but to comply with the CR protocol, humans must cognitively override any feelings of hunger that develop or place themselves into environments where such hunger cannot be acted upon. This is extremely difficult when trying to also live in modern society where food intake has acquired many social functions in addition to obtaining nutrition[18]. Like rodents, physiological signals of satiety in humans are also reduced after restriction[18-20] and success at maintaining diet induced weight loss correlates with the level of self-reported hunger[21,22]. Moreover, desire to eat was increased 23%, fullness decreased 26%, satisfaction of appetite decreased 37% and hunger increased 13% in individuals under CR relative to pre-restriction baseline levels[23]. Understanding the mechanisms underpinning these hunger and appetite effects is hence a key goal because it may suggest strategies by which CR could be imposed while minimising hunger development. This would help individuals to remain on restriction for more protracted periods. On the other hand, there is a hypothesis that the hunger signalling is an integral signalling component mediating the benefits of CR[23] hence minimising it may be counter-productive and maximising it may be a more desirable strategy.

Initially, when animals (and humans) are placed under restriction there is a mismatch between intake and expenditure. This deficit potentially drives hunger signalling in the brain via peripheral hormone levels such as leptin and insulin[17]. However, mice under CR eventually regain energy balance at the reduced intake level and stabilise body mass, fat mass and fat free mass after a period of about 30 days in C57BL/6[24] and 30-50 days in MF1 mice[16,25]. If the hunger was driven only by the energy imbalance, then at this point the hunger would dissipate. However, hypothalamic hunger signals remained stimulated in MF1 mice on 20% CR for up to 100 days, long after energy balance was regained[16]. This elevated hunger drive manifests as subsequent overeating after CR ends, termed post restriction hyperphagia (PRH)[26-28]. If hunger is not driven by energy imbalance, then an alternative is that it is caused by the changes in body composition and associated hormonal changes. If hunger develops in relation to the change in body composition under CR, we would anticipate that PRH would also develop progressively over the first 30 days of restriction. An association between PRH and changes in body composition have been reported in humans[29]. In mice, PRH abated only after the restoration of fat mass and fat free mass deposits during refeeding[26]. It has recently been postulated that a key driver of the motivation to eat is the level of fat free mass and associated resting energy expenditure[30-33]. Since fat free mass declines under restriction this would be expected to lower hunger drive, suggesting that perhaps fat mass is a more potent driver of the PRH response, potentially involving reduced leptin levels[34,35].

This study aimed to evaluate the changes in body mass, fat mass, fat free mass, physical activity, body temperature and energy expenditure of adult male C57BL/6 mice, in response to moderate CR (30%) over short time periods (between 5 and 30 days), and to quantify the relationship between these changes and the strength of the hyperphagia response during subsequent *ad libitum* refeeding for 12 days.

Materials and Methods

ANIMALS, HOUSING AND STUDY DESIGN

Procedures were approved by the University of Aberdeen Welfare and Ethical committee and carried out under UK Home Office (License PPL 60/4366) following the ARRIVE guidelines.

Fourteen-week-old male C57BL/6J mice (n=48; 24 mice x 2 batches; Charles River, Ormiston, UK) acclimated for two weeks. They were maintained at 21±1°C under a 12:12h light-dark cycle, with lights on at 04:00h and a "dawn/dusk" period of 20min. Mice were housed individually with *ad libitum* access to water and a low fat/high carbohydrate diet (D12450B, 70% kcal carbohydrate; 20% kcal protein; 10% kcal fat, Research Diets Inc, USA). Cages were enriched with wood shavings, shredded paper bedding and a semi-transparent red igloo.

After acclimation, the mice were implanted intraperitoneally with transmitters (PDT-4000 E-Mitter; MiniMitter, USA) under general anaesthesia. The transmitters monitored both physical activity (counts) and body temperature (°C) continuously *in vivo* via a radio frequency field to receiver pads (ER-4000 Receiver; MiniMitter) under each cage. Measurements were recorded per minute using the VitalView™ data acquisition system (MiniMitter). Physical activity was summed while body temperature was averaged for each hour throughout the study to show daily, dark-phase and light-phase patterns. Food anticipatory activity was calculated as the sum of activity counts 3 hours prior to food provisioning[36].

Once recovered from surgery, food intake and body mass of the mice were measured daily (15:00 and 16:00hrs) for 12 days of baseline before randomisation into *ad libitum* fed control (n=12) and six CR groups (n=6 in each). Two control mice were paired to each CR group. The CR groups were fed 70% of their individual mean food intake averaged over the last 7 days of baseline for 5, 10, 15, 20, 25 or 30 days, followed by 12 days of *ad libitum* refeeding. The onset of restriction was

staggered to ensure that each CR group and their matched controls went into the Oxymax at exactly two days to the end of CR. To determine digestive efficiency, faeces were collected over the last 7 days of baseline, final 5-7 days of CR, first and last 5 days of refeeding.

BOMB CALORIMETRY

Faeces were weighed to 0.0001g and dried at 60°C. The diet was dried weekly and used to correct food intake to dry matter intake. The gross energy kJ of diet and faecal samples were measured by bomb calorimetry (Parr 6100 calorimeter using a semi-micro-oxygen bomb 1109A, Scientific and Medical Products Ltd, UK) with a minimum of three replicates within ±1.5 RSD% required.

Energy intake was calculated as dry matter intake * gross energy of the diet

Faecal energy = dry faecal mass * gross energy of faeces

Energy assimilated = energy intake - Faecal energy

Apparent energy absorption efficiency = Energy assimilated /energy intake.

DAILY ENERGY EXPENDITURE

To measure their daily energy expenditure, mice were moved to individual cages in an open circuit indirect calorimeter (Oxymax, Columbus Instruments, US). Four animals, (3 CR, 1 control) were measured simultaneously for 1 week (penultimate day of CR through the first 5 days of refeeding). A 24-hr acclimation was allowed. The controls received 10g above their daily FI, while CR mice received about 30g of food each day during refeeding. Uneaten food was removed and weighed daily before providing new food. Water remained available *ad libitum*. The respiratory exchange ratio was calculated as the ratio of carbon dioxide produced (VCO_2 ml min^{-1}) to oxygen consumed (VO_2 ml min^{-1}). Energy expenditure (kJd^{-1}) was computed from the respiratory exchange ratio using the Weir Equation[37].

$$Energy\ expenditure = VO2(3.941 + (1.106\ x\ respiratory\ exchange\ ratio\ ))\ x\ 4.184\ x\ 60\ x\ 24)/1000)$$

DUAL ENERGY X-RAY ABSORPTIOMETRY (DXA)

Fat mass and fat-free mass were quantified using DXA (GE PIXImus2 series densitometers with software version 1.46.007; GE Medical Systems, UK). Measurements were made under general anaesthesia during baseline, penultimate day of CR and day 12 of refeeding. Data were corrected with a machine specific calibration equation that was derived after comparison with Soxhlet fat extraction[38]. Fat free mass was calculated as the difference between body mass and fat mass. The bone mineral content and the bone mineral density of the mice was also measured.

STATISTICAL ANALYSES

The data were analysed in R (v3.4.2 2017-09-28) via RStudio (v1.0.143 2016). Data were arranged into 4 feeding phases i.e. BL (last 7 days of baseline), CR (last 5 days of CR), Early refeeding i.e. first 5 days of refeeding within the Oxymax and Late refeeding i.e. final 5 days of refeeding in the homecage). Days when DXA or movement to and from the Oxymax occurred were excluded from physical activity, food anticipatory activity and body temperature data. Group responses to CR duration were tested by modelling the interaction between groups and feeding phase using a linear mixed effects model (lmer function, lme4 package). Mouse ID was entered as a random intercept to account for repeated measures. Comparisons were made to the controls (n=12) and to their respective baselines. Significance of fixed factors was determined by type-3 F-tests with Satterthwaite approximations for denominator degrees of freedom (anova function, lmerTest package). Where significant interactions were observed, further *post hoc* comparisons were made within each feeding phase (One-way ANOVA and TukeyHSD). Models were validated by plots of standardised residuals to fitted data.

The relationship between torpor occurrence and body mass was obtained by logistic regression (GLM function, family binomial). Group differences in energy expenditure on the last day of CR was analysed by a One-way ANOVA (aov function) with *post hoc* Tukey multiple comparisons (TukeyHSD function). Energy expenditure at early refeeding was analysed by modelling the interaction between group and day (lmer function, lme4 package).

Multiple linear regression was fitted to obtain the correlates of the PRH response during refeeding at the individual level. Predictor variables were days on CR (CR-days), body mass, fat mass and fat free mass losses at the end of CR, light-phase body temperature and log food anticipatory activity averaged over the last 5 days of CR. A significance level of $P<0.05$ was adopted for all the analyses. Model was selected based on the overall P value and that of individual variables.

Results

Results are presented for 45 mice. One transmitter failed on day 13 of CR (15d group). It was therefore excluded from the results on body temperature, physical activity and food anticipatory activity but remained in the other analyses.

ENERGY INTAKE, FAECAL ENERGY, ENERGY ASSIMILATED AND APPARENT ENERGY ABSORPTION EFFICIENCY

The GE content (mean±sd) of the diet was 18.16±0.25 kJg^{-1}. Significant group by feeding phase interaction ($F_{(18,113)}=17.15$, $P<0.001$) and main effect of feeding phase ($F_{(3,\ 113)}=447.77$, $P<0.001$) were observed on energy intake. As expected since we supplied the food, the main effect of restriction group was not significant ($F_{(6,\ 38)}=0.76$, $P=0.61$). Baseline energy intake did not differ between the groups (51.47 ± 0.76 kJd^{-1}, One-way ANOVA, $F_{(6,38)}=1.71$, $P=0.15$). The CR groups had significantly lower energy intake than controls (One-way ANOVA, $F_{(6,38)}=34.21$, $P<0.001$) and their own baseline ($P<0.001$) during restriction. All CR groups displayed marked hyperphagia on the first day of refeeding (Figure 1a). Energy intake was significantly higher than controls (except the CR group 5d) during early refeeding

(first 5 days) (One-way ANOVA, $F_{(6, 38)}$=8.95, P<0.001). Energy intake returned to baseline levels and was not different from the controls during late refeeding (One-way ANOVA, $F_{(6,38)}$=0.72, P=0.639, Figure 1a).

Faecal energy and energy assimilated varied in direct proportion to the EI in each feeding phase. There was a significant feeding phase effect on apparent energy absorption efficiency ($F_{(3,112)}$=8.56, P<0.001) but no effect of restriction group ($F_{(6,38)}$=1.70, P=0.15) nor interaction ($F_{(18,112)}$=1.22, P=0.26). Apparent energy absorption efficiency at early refeeding (93.13±0.11%) was significantly higher than the other 3 phases, particularly in the 10d and 30d CR groups.

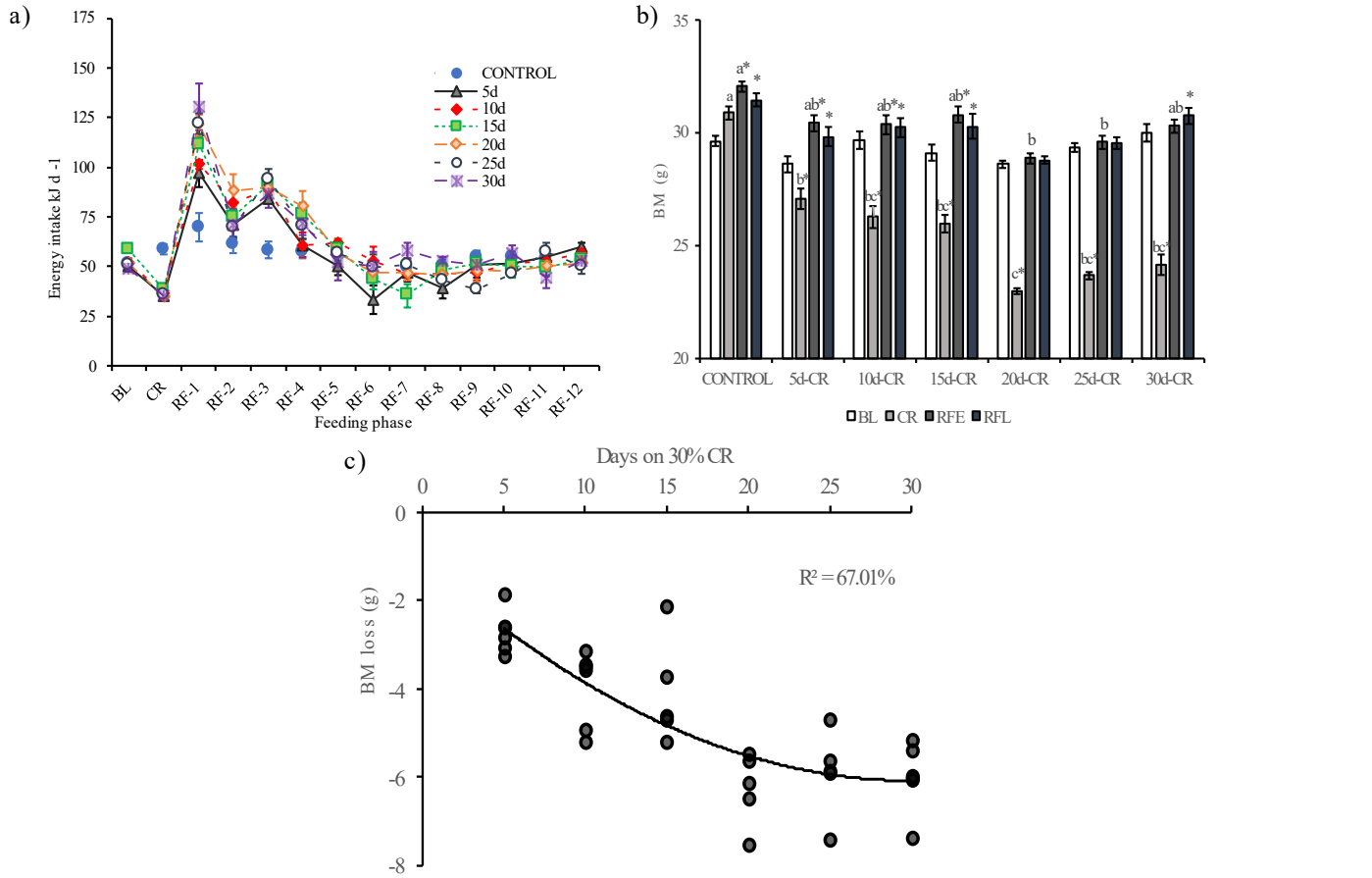

Figure 1: Pattern of post restriction hyperphagia (PRH) response. Changes in food intake and body mass (BM) following PRH in 30% calorie-restricted mice (30CR) for 5, 10, 15, 20, 25 and 30 days contrasted with ad libitum fed controls. a) Energy intake (mean ± SEM) for 7 days of baseline (BL), last 5 days of CR (CR) and 12 days of refeeding (RF1–RF12). b) Average BM (g) over BL, CR, first 5 days of refeeding (RFE) and last 5 days of refeeding (RFL). c) Individual BM loss decreased with the duration of CR up to 20 days. Different superscripts represent significant differences between the groups within a feeding phase (P<0.05). * Represents significant differences (P≤0.001) from the respective baselines of each group.

BODY MASS

Significant group by feeding phase interaction on body mass ($F_{(18,924)}$=116.87, P<0.001) was observed. The main effects of group ($F_{(6,38)}$=2.59, P=0.033) and feeding phase ($F_{(3,924)}$= 1737.93, P<0.001) were also significant. Baseline body mass did not differ between the groups (ANOVA $F_{(6,38)}$=0.37; P=0.894, Figure 1b). All the CR groups lost body mass and weighed significantly less than the controls (ANOVA $F_{(6,38)}$=13.29; P<0.001, Figure 1b). Compared to baseline, controls gained body mass and were significantly heavier through the feeding phases. Individual body mass loss increased with the duration of CR up to 20 days (Non-linear regression, $F_{(2,30)}$=30.47, P<0.001; Figure 1c). The CR groups regained body mass from the first day of refeeding and were fully recovered to the level of controls by end of refeeding (ANOVA $F_{(6,38)}$=2.39 and 1.06; P=0.047 and 0.406 for early and late refeeding respectively; Figure 1b). The CR groups 5d, 10d, 15d and 30d regained body mass above their respective baseline body masses through refeeding, while mice on CR for 20d and 25d did not differ significantly from their baseline (Figure 1b).

FAT MASS AND FAT-FREE MASS

Significant group by feeding phase interaction ($F_{(12,75)}$=4.14, P<0.001) and main effect of feeding phase ($F_{(2,75)}$=54.33, P<0.001) but no effect of group ($F_{(6,38)}$=1.24, P=0.311) was observed in fat mass. Baseline fat mass did not differ significantly between the groups (4.52±0.18g, One-way ANOVA $F_{(6,38)}$=0.21; P=0.972, Figure 2a). CR groups lost fat mass (but only the 20 and 25d were significantly lower than the controls) (One-way ANOVA $F_{(6,37)}$=3.95, P=0.004, *post hoc* TukeyHSD, P=0.008 and 0.010 for 20 and 25d respectively; Figure 2a). By the end of refeeding, all CR groups had regained fat mass and were not different from the controls (One-way ANOVA $F_{(6, 38)}$=1.07; P=0.400; Figure 2a).

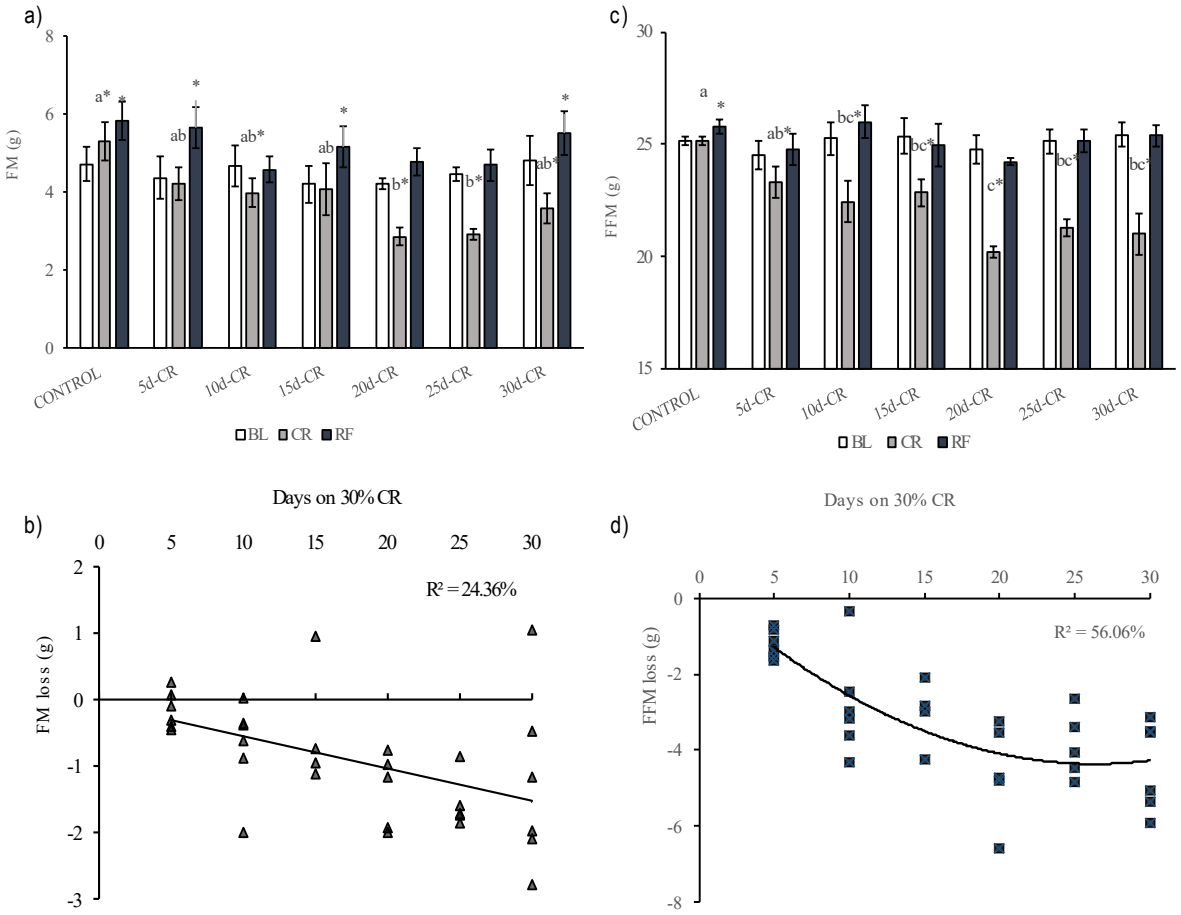

Figure 2: a) Fat mass (FM) (g) and (c) Fat-free mass (FFM) (g) of ad libitum fed controls and 5, 10, 15, 20, 25 or 30 days 30% calorie-restricted (CR) mice. Measurements were taken on the last day of baseline (BL), penultimate day of CR (CR) and the last day of refeeding (RF). b) FM loss (g) over CR decreased linearly while d) FFM loss (g) decreased non-linearly with days on CR. Superscripts represent significant group differences within a feeding phase ($P<0.05$). * Represents significant differences from the respective group baseline ($P<0.05$). Data is mean ± SEM.

Compared to baseline, the controls significantly gained fat mass by 23.7% at the end of refeeding. fat mass of the CR groups, except groups 5d and 15d, were significantly lower than their respective baselines during restriction. However, by the end of refeeding, fat mass of the 5, 15 and 30d was significantly higher than their respective baselines, while the 10, 20 and 25d groups returned to baseline levels (Figure 2a). The duration of CR explained about 24.4% of the variability in individual fat mass loss (Linear regression, $F_{(1,30)}=9.66$; $P=0.004$, Figure 2b).

With respect to fat free mass, significant effects of group by feeding phase interaction ($F_{(12,75)}=15.60$, $P<0.001$), and feeding phase ($F_{(2,75)}=254.03$, $P<0.001$) were observed, but the overall group effect marginally failed to reach significance ($F_{(6,38)}=2.21$ $P=0.063$). Baseline fat free mass was not different between the groups (25.10 ± 0.19g, One-way ANOVA $F_{(6,38)}=0.38$, $P=0.888$; Figure 2c). Controls maintained fat free mass during the CR phase, but this increased significantly at the end of the refeeding phase. During restriction, the fat free mass of CR groups (except 5d) was significantly lower than controls (One-way ANOVA $F_{(6,37)}=10.29$, $P<0.001$) and their baseline (Figure 2c). The CR groups regained fat free mass to the level of the controls by the end of refeeding ($F_{(6,38)}=1.15$, $P=0.355$, Figure 2b). Individual fat free mass declined non-linearly with more days on CR ($F_{(2,29)}=18.49$, $P<0.001$; Figure 2d).

The bone mineral content did not differ between the groups or across the feeding phases ($F_{(12,76)} = 1.23$, $P = 0.282$. Using a general linear model, logit link, family (quasibinomial), no significant difference was observed in the probabilities of obtaining the bone mineral densities between the groups and across feeding phases (Group = $F_{(6, 128)} = 0.017$, $P = 0.292$; Feeding phase = $F_{(2, 126)} = 0.004$, $P = 0.288$).

PHYSICAL ACTIVITY AND FOOD ANTICIPATORY ACTIVITY

All mice displayed peak activity in the dark phase and low activity in the light phase. Total physical activity showed a significant group by phase interaction ($F_{(18,31)}=2.22$, $P=0.024$), and main effect of feeding phase ($F_{(3,31)}=7.40$, $P<0.001$) but no effect of CR duration ($F_{(6,37)}=0.39$, $P=0.882$). Total physical activity did not differ between the groups at baseline physical activity (17158±314), CR and early refeeding. Significant differences were observed during late refeeding with the 20d significantly higher than controls (One way-ANOVA $F_{(6,37)}=2.91$, $P=0.020$, Figure 3a). Compared to their baselines, *ad libitum* fed controls and CR groups 5, 10 and 15d did not alter their total physical activity across all the feeding phases (all $P>0.05$; Figure 3a). Significantly higher physical activity compared to baseline were observed in early refeeding phase of the 30d, the late refeeding phase of the 20d and 25d and CR phase of the 25d ($P<0.05$). (Figure 3a).

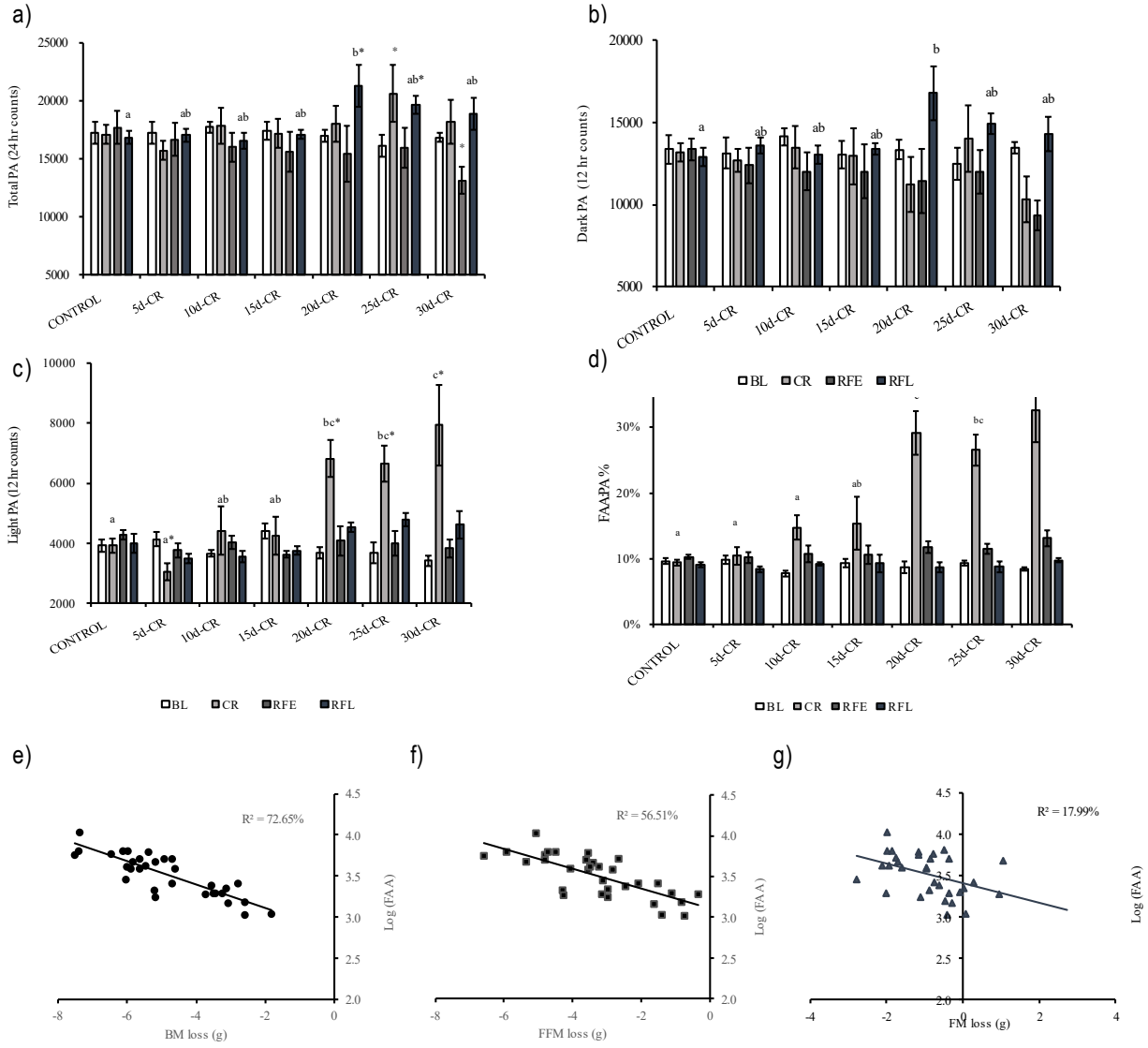

Figure 3: Physical activity (PA) of ad libitum fed controls and 5, 10, 15, 20, 25 or 30 days 30% calorie-restricted (CR) mice. a) Total PA counts per day; b) Dark-phase PA; c) Light-phase PA d). Food anticipatory activity (FAA %). PA was averaged (mean ± SEM) over the last 7 days of baseline (BL), last 5 days of CR, first 5 days of refeeding (RFE) and last 5 days of refeeding (RFL). a, b indicate significant differences within a feeding phase ($P<0.05$). * Represents significant differences in activity from the respective baselines ($P<0.05$). e-g) FAA increased relative to e) body mass (BM) loss f) fat-free mass (FFM) loss and g) Fat mass (FM) loss.

Separating the dark and light phases, there was no significant group by feeding phase interaction ($F_{(18,14)}=2.02$, $P=0.091$) nor main effect of group ($F_{(6,37)}=0.50$, $P=0.804$) in dark-phase physical activity, but a significant feeding phase effect was observed ($F_{(3,14)}=8.74$, $P<0.001$). Across the groups, dark-phase activity during early refeeding was significantly lower than at baseline ($P<0.01$) while late refeeding was significantly higher than during CR and early refeeding ($P<0.01$) (Figure 3b). With respect to light-phase activity, significant group by feeding phase interaction ($F_{(18,133)}=6.24$, $P=0.001$), main effects of group ($F_{(6,37)}=4.16$, $P=0.003$), and feeding phase ($F_{(3,133)}=20.51$, $P=0.001$) were observed. The average light-phase activity across all individuals at baseline was 3831±90 counts/12 hours (about 22.5% of the total physical activity). During CR, light-phase activity almost doubled in the 20d, 25d and 30d groups and was significantly higher than controls, the 5d (all $P<0.05$), and their respective baselines (Figure 3c). In contrast the light-phase activity of 5d was lower than baseline ($P=0.047$) and10d and 15d unaltered (Figure 3c). Upon refeeding, their light-phase activities returned to baseline levels and they did not differ significantly from controls at early or late refeeding phases ($P>0.05$, Figure 3c).

Food anticipatory activity increased in response to the duration of CR. Over baseline, food anticipatory activity remained low with no differences between the groups (1552±40 counts/3hrs; $F_{(6,37)}=1.46$, $P=0.219$; Figure 3d). During CR, groups 20d, 25d and 30d significantly increased food anticipatory activity (5150±470, 5013±394 and 5797±1098 counts/3hrs respectively) compared to controls, and respective baselines (all $P<0.001$). Food anticipatory activity returned to baseline levels following refeeding. Food anticipatory activity varied relative to individual body mass ($R^2=72.65\%$), fat free mass ($R^2=56.51\%$) and fat mass ($R^2=17.99\%$) losses during restriction (Figure 3e, f, g).

BODY TEMPERATURE AND TORPOR

Body temperature followed a diurnal rhythm, peaking in the active dark phase and lowest in the resting light phase. Overall, significant group by feeding phase

interactions ($F_{(18,111)}$=13.91, 9.13 and 13.71, P<0.001), effects of CR duration ($F_{(6,37)}$=7.06, 5.78 and 5.03, P<0.001) and feeding phase ($F_{(3,111)}$=213.01, 114.98 and 249.37, P<0.001) were observed for the daily, dark-phase and light-phase body temperature respectively. Baseline body temperature was similar between all the groups with mean daily body temperature (36.5±0.02°C), dark-phase body temperature (37.2±0.03°C) and light-phase body temperature (35.7±0.04°C) (Figure 4a). During restriction, the CR groups significantly decreased their daily, dark-phase and light-phase body temperature (all P<0.001; Figure 4a). At least one mouse in each CR group (except 10d) showed a torpor response i.e. body temperature below 31°C during CR (Table 1). Torpor was significantly related to individual body mass during restriction (GLM-Binomial, $\chi^2_{(1)}$=158.83, P<0.001). Upon refeeding, daily, dark-phase and light-phase body temperature of CR groups did not differ significantly from controls (all P>0.05, Figure 4a).

Table 1: Torpor response of mice on 30% calorie restriction (CR) for 5, 10, 15, 20, 25 or 30 days. Torpor was defined as a state of decreased body temperature below 31°C. At least one mouse in each CR group (except the 10d) showed a torpor response.

Mouse id	Group	First incidence of torpor (CR-DAY)	No of torpor days	Total time in torpor (mins)	Lowest recorded body temperature (°C)
1	5d	5	1	82	29.64
8	15d	8	3	341	26.12
16	20d	12	8	1084	26.09
43	20d	14	5	468	27.40
18	20d	20	1	210	26.21
39	25d	10	9	649	27.64
44	25d	13	4	258	27.62
10	25d	13	11	1048	25.59
34	25d	22	2	140	29.24
2	25d	25	1	226	25.89
14	30d	14	17	4502	23.97
40	30d	17	8	737	26.24

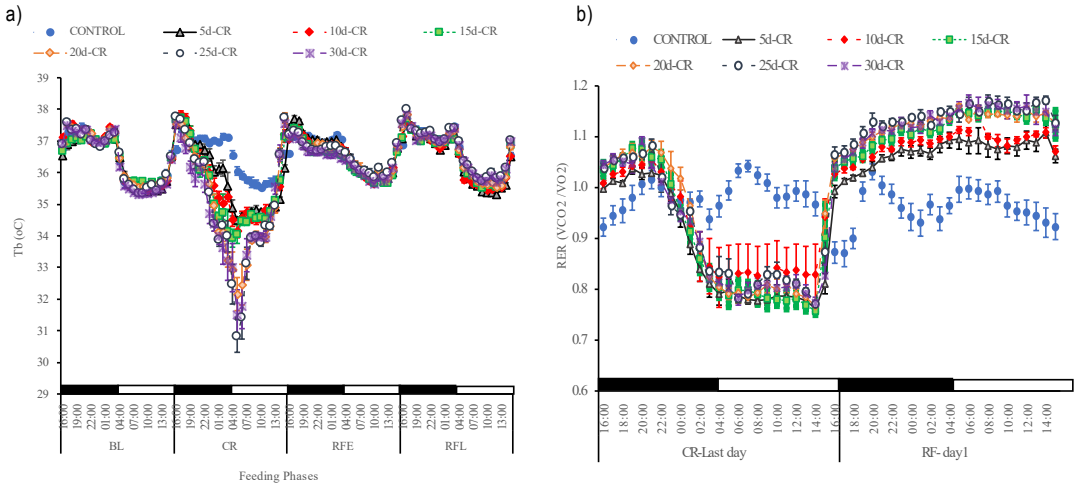

Figure 4: a) Body temperature (T_b °C) and b) Respiratory exchange ratio (RER) of ad libitum fed controls and 30% calorie-restricted (CR) mice for 5, 10, 15, 20, 25 or 30 days at different feeding phases. Dark-phase (16:00 – 03:59 hrs) and light-phase (4:00 – 15:59hrs) T_b was averaged each hour over the last 7 days of baseline (BL), last 5 days of CR, first 5 days of refeeding (RFE) and last 5 days of refeeding (RFL). RER is presented on the last day of CR and first day of refeeding. Data is mean ± SEM.

RESPIRATORY EXCHANGE RATIO AND ENERGY EXPENDITURE

Mice utilised different fuel sources for their energy expenditure during CR. mainly in the light-phase when no food was available. Light-phase respiratory exchange ratio averaged over all the CR mice was 0.80±0.004 on the last day of restriction. Mean daily respiratory exchange ratio of the CR mice rose to 1.11±0.006 through the first three days of refeeding, then decreased to 0.94±0.08 on days 4 and 5. Controls maintained an average daily respiratory exchange ratio of 0.95±0.01 (Figure 4b).

The energy expenditure of the CR groups decreased significantly below the controls during restriction (P<0.001, Figure 5a,b,c). Upon refeeding, energy expenditure increased and was not different from the controls over early refeeding (Figure 5a,b,c). There was a significant group by day interaction in daily energy expenditure during refeeding ($F_{(6,172)}$=6.28, P<0.001). The controls and 5d group maintained their daily energy expenditure from the first day while the other CR groups significantly increased their daily energy expenditure with each day of refeeding.

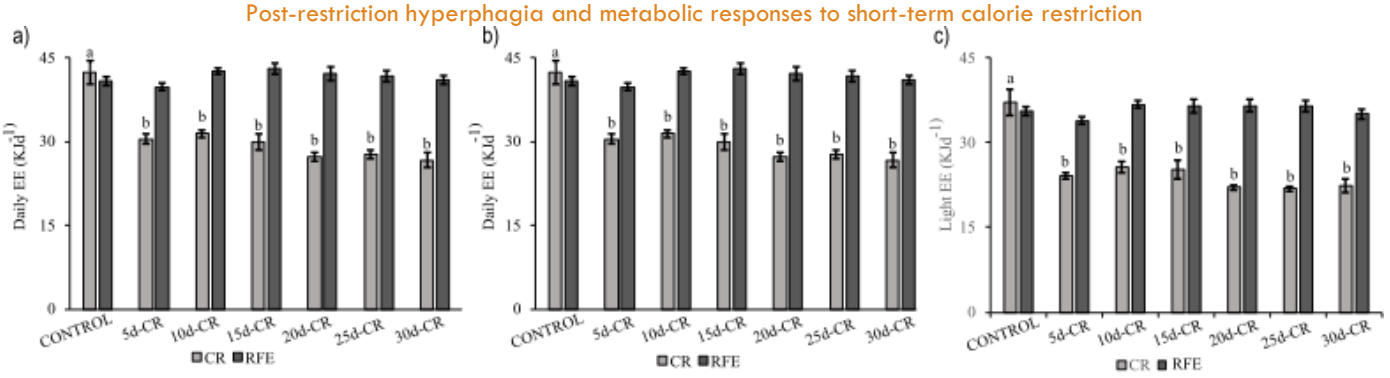

Figure 5: a) Daily (24 hrs average); b) Dark-phase (12 hrs, 16:00 – 3:59); c) and Light-phase (12 hrs, 4:00 – 15:59hrs) energy expenditure (EE) of ad libitum fed controls and 30% calorie restricted mice (CR) for 5, 10, 15, 20, 25 or 30 days. EE (mean ± SEM) was measured on the last day of CR and averaged over the first 5 days of refeeding (RFE).

Post-restriction hyperphagia (PRH)

PRH was observed in all CR groups with peak energy intake on the first day of refeeding. Energy intake fell on day 2, increased slightly on day 3, followed by a gradual decrease towards baseline levels from day 4 of refeeding (Figure 1). Energy intake of the CR groups (except 5d) on RF-day1 and their total energy intake over RF-days 2-4 was higher than the controls (One-way ANOVA $F_{(6,38)}$=9.83 and 4.31 P<0.001 and =0.002 respectively).

At the individual level PRH (energy intake on refeeding day 1) was correlated with duration on restriction (r=0.6; R^2=39.6%, $F_{(1,30)}$=19.68, P<0.001). Other predictors of peak PRH on RF-day1 were body mass loss (R^2=42.7%, P<0.001, Figure 6a); FFM loss (R^2=26.5%, P=0.003, Figure 6b); fat mass loss (R^2=14.3%, P=0.033, Figure 6c); Log(FAA) (R^2=44.6%, P<0.001); and light-phase body temperature (R^2=32.1%, P=0.001). These predictor variables were all strongly correlated with each other (Figure 6d, Figure S4). Overall, more days on CR was associated with a greater body mass loss. Body mass loss was correlated with decreased light-phase body temperature and a higher FAA. FAA was associated with greater energy intake on RF-day1. FFM loss was the main component of body mass that drove the PRH (Multiple regression R^2=32.7%, fat mass P=0.036, FFM P=0.003). Beyond the first day of refeeding, there were no significant predictors of PRH for the following refeeding days. However, energy intake on RF-days 1 and 2 were inversely associated such that mice with a higher energy intake on day1 had a lower energy intake on day 2 (r=-0.5, R^2=23.2%, P=0.005; Figure 6d and 7)

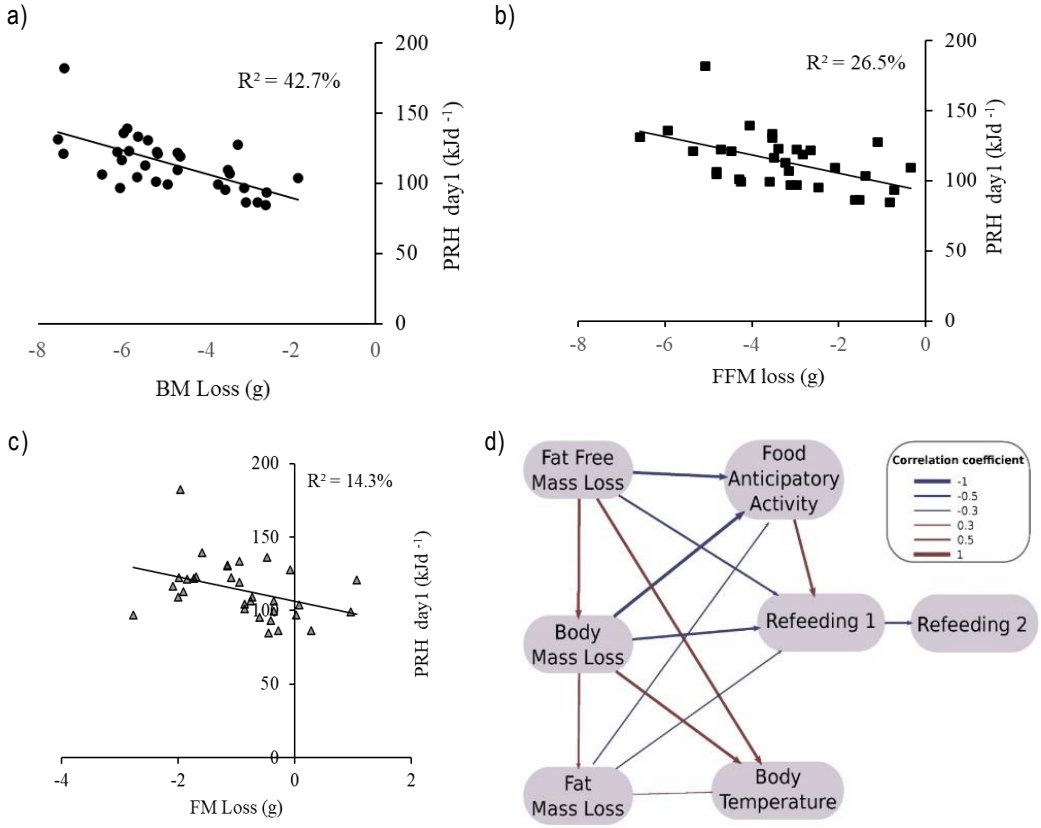

Figure 6: Post restriction hyperphagia (PRH, kJday-1) on day 1 of refeeding was predicted by a) body mass (BM) loss (P =0.001); b) fat-free mass (FFM) loss (P = 0.003) and c) Fat mass (FM) loss (P = 0.033) in mice 30% calorie-restricted for 5, 10, 15, 20, 25 or 30 days. d) Correlation structure between predictor variables and PRH on days 1 and 2 of refeeding. Brown lines represent positive correlation while blue lines depict negative correlation. All variables were correlated with peak PRH on day 1 of refeeding. None of the variables could explain energy intake on day 2 of refeeding.

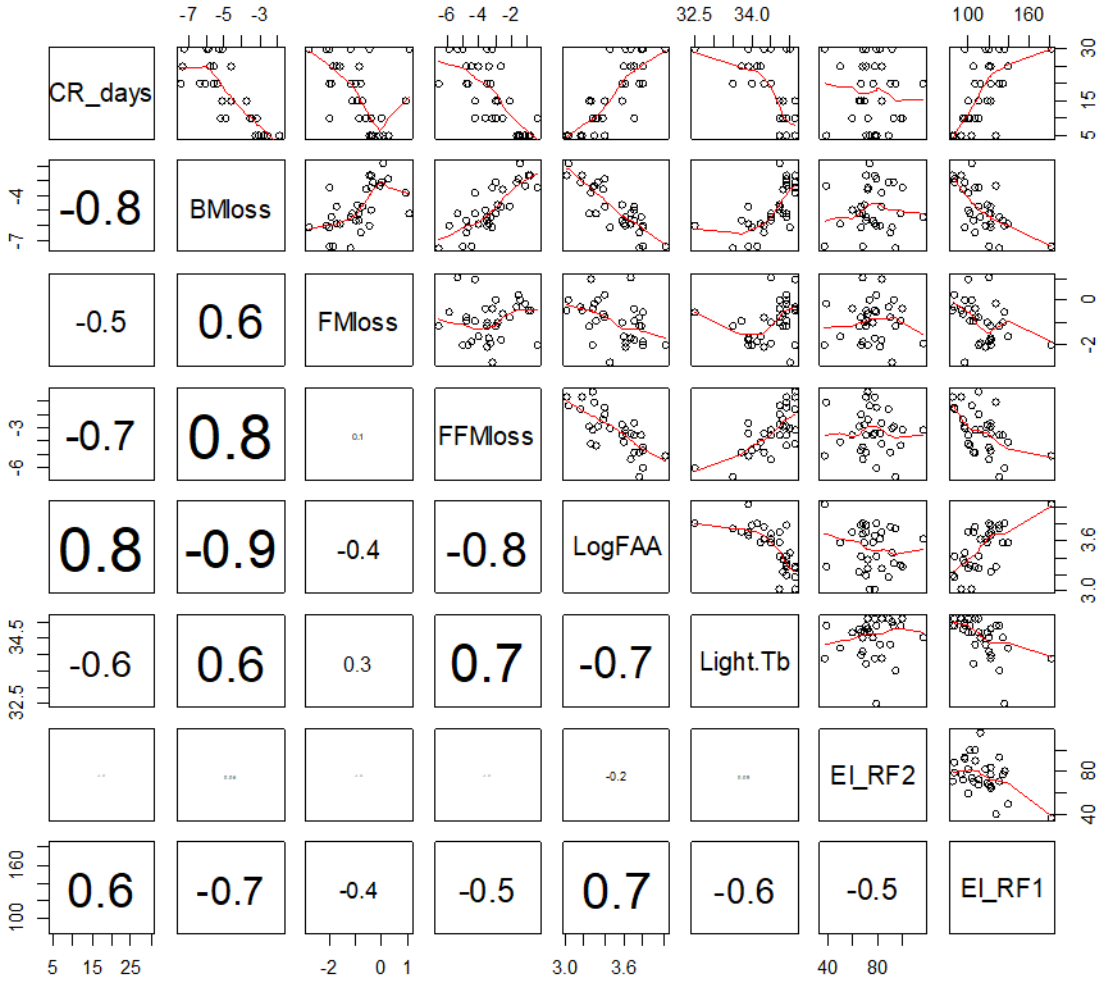

Figure 7: Pair plots and correlations between predictor variables and post restriction hyperphagia (PRH) on days 1 and 2 of refeeding. All variables were correlated with PRH on day 1 of refeeding (Energy intake EI-RF1) but not with day 2 of refeeding (EI-RF2).

Discussion

The study examined the metabolic responses to 30% CR for 5 to 30 days in adult male mice, and their relationship to post-restriction hyperphagia (PRH) as a measure of hunger. We studied only males. Previous work has indicated that the responses to CR in terms of lifespan effects of males and females are similar[6]. However, there are some sex differences in the physiological and morphological effects of short-term CR. For example, the impact of restriction on body composition appears altered, with effects on fat mass being more blunted or even absent in females[39,40]. Whether this difference in body composition responses impacts the PRH responses is currently uncertain. However, some work has suggested that while females may lose less fat under-restriction they also gain less upon refeeding[40]. Future work may address how these differences between the sexes and individuals are mediated in terms of differences in the level of PRH and other metabolic responses. In males, short-term CR resulted in an energy deficit with downstream changes in body mass, body composition, light-phase activity, body temperature and energy expenditure. Peak PRH observed on the first day of refeeding was strongly related to individual body mass loss over the period of restriction, of which fat free mass loss was the main component. This response contrasts the individual responses of striped hamsters to restriction, where the ones that lost least fat were actually the individuals that gained most fat on refeeding[41]. The first 30 days of CR represent the dynamic phase of rapid body mass loss in C57BL/6 mice[24] evident in the current study as body mass loss which proceeded in a nonlinear fashion with the greatest declines during the first 15 days. Fat free mass loss rather than fat mass loss was the primary contributor to body mass loss in the current study. On the other hand, fat mass was the main component of BM gains above baseline, during refeeding. Similar effects were also observed in tree shrews (Tupaia belangeri)[42] and humans[43]. This process has been called 'collateral fattening' whereby fat mass gains accompany the restoration of fat free mass after weight loss and has been previously reported in human studies of CR[27]. It is thought to be related to the initial fat mass: fat free mass ratio (FM:FFM), such that obese subjects with a high (FM:FFM) ratio have a greater proportion of their BM loss as fat mass while lean subjects with low initial fat mass stores have higher fat free mass losses during CR[27,44]. Deficits in fat free mass result in lower energy expenditure and activate a feedback loop that drives energy intake to restore fat free mass, with resultant increase in fat mass[44]. Both fat mass and fat free mass have been shown to individually predict the PRH response in previous CR studies on mice[26] and humans[29]. In the current study, fat free mass loss was a stronger predictor of the individual PRH. Because the balance of loss of fat-

free mass and fat mass differs with sex then this may affect the level of PRH in females. Fat free mass is positively associated with daily energy intake in conditions of energy balance in humans[31] and mice[45]. This association is posited to be indirect, mediated by the action of fat free mass on the resting metabolic rate[27,31,45]. In conditions of negative energy balance though, it has been suggested that the loss of fat free mass actively drives the hyperphagic response[31] by feedback signals from fat free mass (such as myokines) as the body tries to replete fat free mass stores[27]. If individuals gain substantially more fat mass as they attempt to replete fat free mass, that could lead to 'collateral fattening' which has implications for normal weight individuals attempting weight loss by CR, as they may be setting themselves up to gain more fat if they discontinue engaging in the CR protocol. Moreover, this might suggest that repeated cycles of restriction and refeeding would lead to a progressive increase in body fatness[46]. However, a study of repeated cycling between restriction and re-feeding compared to just ad libitum food intake in the same C57BL/6 mice as used here did not detect any significant elevation in fatness for the fasted-refed individuals[47].

Some previous studies reported no effect of CR duration on PRH[26,48] The study of mice[26] compared mice exposed to CR for durations of 25 and 75 days. Over this interval the change in body weight, and hence likely body composition changes are relatively small, consistent with the idea that PRH develops over the first 30 days due to changes in body composition. The study of rats by contrast compared responses to restriction for either 3 or 5 days and also showed no difference in the response likely because the changed body composition over this interval was not different. Interindividual variability in response to CR reported in animal and human studies[36,49] may reflect intrinsic baseline characteristics and/or flexibility in the adaptive strategy adopted[36]. For example, rats that were artificially selected for their high aerobic capacity displayed greater activity and body mass loss than their low activity counterparts when exposed to 50% CR for 3 weeks[50]. Furthermore, non-gorging mice were less active and resistant to weight loss whereas mice that gorged were more active, displayed higher food anticipatory activity, lost more weight and ate more when exposed to the same level (25%) of CR[51]. Desert golden spiny mice exposed to 50% CR for 40 days employed either a "resistant" strategy (low activity levels, energy expenditure and little body mass loss) or "non-resistant" strategy (high activity, energy expenditure and body mass loss)[52]. These responses contrast the individual responses of striped hamsters to restriction, where the ones that lost least fat gained the most fat on refeeding[41]. The findings of the current study suggests that the duration of CR influences PRH indirectly, depending on the individual extent of compensatory responses recruited. This individual variability in the CR response and hence impact on hunger responses should potentially be considered when deciding in which individuals CR might be successful, as a "one size fits all" approach may not suffice.

In this study, body temperature was negatively associated with days on CR and body mass loss, with a greater tendency to use torpor in the 20, 25 and 30d CR groups. Lowered body temperature has been suggested to be a contributor to the positive energy balance during re-feeding leading to catch-up fat[53]. However, in our study, lower body temperature between the CR groups did not translate to differences in their energy expenditure on the last day of CR. The early torpor response of some individuals may indicate higher physiological flexibility whereby they adjusted quickly to the energy deficit and employed torpor as an energy conservation strategy[54,55]. The ability of CR mice to utilise different fuel sources for their energy expenditure was reflected in the changes in the respiratory exchange ratio – lipid utilisation from fat reserves during restriction, overfeeding and possible lipogenesis in the first 3 days of refeeding and a shift to carbohydrate utilisation thereafter. In our study we maintained the mice on a constant low fat diet. Other studies have varied the dietary composition during refeeding and shown that intake is exaggerated, and fat gain magnified when the mice are fed a high fat diet during the re-feeding phase[56]. In contrast a high protein diet during refeeding blunts the fat accumulation[57]. Cold exposure also prevents fat accumulation during refeeding in striped hamsters[58]. During peak lactation in mice food restriction was not followed by hyperphagia relative to lactating controls during the refeeding phase, presumed to be because at peak lactation animals are already at their maximal levels of food intake[59].

CR mice did not conserve energy by reducing total physical activity in this study. Changes in their temporal activity patterns and increased food anticipatory activity are consistent with previous reports[60]. CR as well as temporal restriction (without CR) induce food anticipatory activity[61-63]. Food anticipatory activity is akin to the increased foraging activity or migration demonstrated by animals exposed to food shortages under natural conditions[52,55]. It has been shown to contribute to skeletal muscle strength and improved bone mineral density in rodents on CR[64], though bone mineral density was not different between CR levels in this study. The positive association between food anticipatory activity and PRH at the individual level in the current study implies that food anticipatory activity could serve as a proxy measure of hunger levels in rodents. Food anticipatory activity correlated strongly with the expression of orexigenic hypothalamic neuropeptide Y (NPY) and Agouti related-protein (AgRP) in mice on graded levels of CR[17].

Conclusion

In conclusion, short term CR activates compensatory responses and acute post-restriction hyperphagia upon refeeding in mice. The peak hyperphagia response was significantly related to individual body mass loss, fat free mass loss, fat mass loss, body temperature and food anticipatory activity. fat free mass loss was the main factor that predicted PRH. This effect is somewhat inconsistent with the idea that higher rather than reduced fat free mass and RMR is a key driver of appetite. Understanding the signals emanating from fat free mass that might drive this response should be a key goal allowing the potential development of adjunct therapies that might supplement traditional CR approaches. The impact of sex on these responses may be different and should be a topic for further study.

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Author contributions: JRS conceived the study. JRS, SEM, CH designed the experiments. SEM was the Home Office Project License holder and performed the surgeries. EPU, SEM, CH performed the experiments. EPU analysed the data and drafted the paper. JRS, SEM, CH contributed to analysis and interpretation of the results and reviewed drafts. All authors have read the paper.

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