Intermittent protein restriction elevates food intake and plasma ghrelin in male mice

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Declaration of Interest

None

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Abstract

Low-protein diets affect body weight, body composition, food intake, and food preferences in mice. Furthermore, single periods of protein restriction can have lasting effects on these parameters. We sought to examine the effect of multiple, short, bouts of protein restriction, relative to long-term maintenance on either a control (NR) or protein-restricted (PR) diet. We found that male mice experiencing intermittent protein restriction (IPR) were indistinguishable from NR mice in terms of body weight and composition, but had food intake and plasma ghrelin as high as mice on PR diet, even when they were returned to control diet. This was not found in female mice. The results of this experiment highlight the importance of diet history on food intake and ghrelin levels in male mice, and the difference in how PR diet affects male and female mice.

1. Introduction

Low-protein diets have wide-ranging effects on physiology and ingestive behavior. In male mice, low-protein diets often lead to a reduction in body weight, relative to mice eating diets that are sufficient in protein (Fontana et al., 2016; Green et al., 2022; Hill et al., 2019, 2022; Laeger et al., 2014; Larson et al., 2017; Trautman et al., 2023; Wu et al., 2021), despite increasing food intake (Fontana et al., 2016; Green et al., 2022; Hill et al., 2019; Laeger et al., 2014; Trautman et al., 2023; Volcko & McCutcheon, 2022). This mismatch appears due to increased energy expenditure observed in mice on low-protein diets (Hill et al., 2019, 2022; Wu et al., 2021). Low-protein diets also affect body composition. Some studies report a reduction in both fat and lean mass (Fontana et al., 2016; Hill et al., 2022; Larson et al., 2017; Trautman et al., 2023; Wu et al., 2021), some in only lean mass (Green et al., 2022), while others have found increases in fat mass (Simpson et al., 2013; Sørensen et al., 2008). Female mice have been studied far less frequently, but low-protein diets do not seem to have as large of an effect, if any, on body weight or body composition (Blais et al., 2018; Green et al., 2022; Larson et al., 2017; Volcko & McCutcheon, 2022); some studies do not find an effect of low-protein diet on food intake in female mice (Green et al., 2022; Larson et al., 2017) while others find increased intake (Blais et al., 2018; Volcko & McCutcheon, 2022).

Food choice is different in mice eating diets sufficient in protein compared to those eating a low-protein diet. Specifically, low-protein diets cause rodents to prefer sources of protein, such as the milk protein casein, over sources of carbohydrate, such as maltodextrin (Chiacchierini et al., 2021; Hill et al., 2019; Murphy et al., 2018; Volcko & McCutcheon, 2022). Despite differences between males and females in metabolic responses to low-protein diets, we have recently shown that this heightened protein preference is nearly identical in male and female mice (Volcko & McCutcheon, 2022).

The effects of low-protein diets can persist long after the animals are eating a normal diet again. For example, offspring of dams fed a low-protein diet during pregnancy and lactation have higher food intake even at a year of age (Qasem et al., 2016), and rats fed a low-protein diet during adolescence or early adulthood and a normal-protein diet thereafter, show elevated food intake and higher fat mass in adulthood than rats who did not experience a period of protein restriction (de Oliveira et al., 2013; Malta et al., 2014). The same group, however, did not find an effect when the diet manipulation occurred later in adulthood (Malta et al., 2016), indicating that the age of the rats was critical in determining the long-term effects of a period of protein restriction.

If a single period of protein restriction, in some developmental windows, can affect later behavior and body composition, one may also wonder if repeated bouts of protein restriction have

an effect. This was explored in a recent study by Torres et al. (2022), in which individual male mice alternated between control and low-protein diets, with varying times on each diet (from 3 - 9 weeks). This study found that mice regulated body weight and food intake depending on which diet they were eating (i.e. eating more but gaining less weight when on the low-protein diet compared to the control diet) (Torres et al., 2022). Because this was a single-case experimental design, however, it did not examine if repeated periods of protein restriction would have a long-term effect on body weight and composition compared to if no restriction was experienced. We were curious to know what would happen to food intake, body weight and composition, and ghrelin levels if mice alternated between control and protein-restricted diets every seven days, and how this would compare to mice with continuous access to either NR or PR diet.

2. Methods

2.1 Animals

The experiment was conducted on two cohorts of mice. The first cohort consisted of forty-two adult male C57BL/6NRj mice, and the second cohort of thirty-six female C57BL/6NRj mice (6 – 8 weeks old). All mice were purchased from Janvier (France) and housed in a temperature- and humidity-controlled room, maintained on a 12:12 light:dark cycle (lights-on at 07:00). Two mice shared each cage, separated by a perforated divider that allowed visual, olfactory, and auditory communication but prevented major physical contact, and allowed individual measurements of food intake. Water and food were available *ad libitum*. A third of the mice were fed a control diet (20% casein; D11051801; Research Diets), a third of the mice an isocaloric protein-restricted diet (5% casein; D15100602; Research Diets), and a third of the mice alternated between the two diets. The three groups, each with 12-14 mice, were therefore the non-restricted controls (NR), the chronically protein-restricted (PR), and the intermittently protein-restricted (IPR). All mice were fed the control diet for a week at the start of the experiment (Fig 1A). Both mice from each cage experienced the same diet condition. All animal care and experimentation followed the EU directive 2010/63/EU for animal experiments, and was approved by the National Animal Research Authority in Norway.

2.2 Body weight and food intake

Body weight was measured three days per week. For male mice, food intake was measured two days per week: one day immediately before the IPR group changed diets, and one day immediately after the IPR diets were switched. These two measurements were averaged for an average intake per week. For female mice, food intake was measured weekly.

105 *2.3 Body composition*

106 Body composition was assessed at baseline (when all mice were on control diet) and after
107 8 weeks (at the end of the experiment). Mice were weighed early in the light phase, and body
108 composition examined 0-6 hours later in an echoMRI-700 Body Composition Analyzer (echoMRI,
109 Houston, TX, USA), with an equal number of mice from each diet condition measured earlier and
110 later in the day.

111

112 *2.4 Plasma levels of ghrelin*

113 At the end of the experiment, plasma was collected, with half the mice in each diet group
114 killed at each of two time points. In the "fasted" condition, plasma was collected at the beginning
115 of the dark phase / end of the light phase with food removed 4-6 hours beforehand. In the "fed"
116 condition, plasma was sampled at the beginning of the light phase / end of the dark phase. At the
117 time of plasma collection, mice in the IPR group had switched back to NR diet 7 days earlier. Mice
118 were killed by decapitation under isoflurane anesthesia and trunk blood was collected in K_2 EDTA-
119 coated tubes (16.444.100, Sarstedt) with 1 mg/ml AEBSF (Merck SBR00015) and placed on ice.
120 Blood was then centrifuged, within an hour of collection, for 10 minutes at 3000 rpm and 4°C
121 (Mikro 220R centrifuge). Plasma was separated, acidified with hydrochloric acid to a concentration
122 of 0.05N, and frozen at -70°C until analysis. Active ghrelin was assessed by ELISA (Millipore
123 EZRGRA-90K) on a ClarioStar microplate reader (BMG Labtech).

124

125 *2.5 Data analysis*

126 Statistical analyses were conducted using JASP (https://jasp-stats.org/). Post-hoc tests
127 were all Tukey corrected.

128 Body weight per week for each mouse was an average of three measurements. A mixed
129 model ANOVA was conducted, with Group (NR, PR, or IPR) as between-groups factor and Time
130 as within-groups factor. Body composition measures were mixed-design ANOVA with Group and
131 Time (baseline vs end of experiment) as factors.

132 Food intake per week for each mouse was an average of two measurements for males,
133 and a single weekly measurement for females. Values over 5 g per day, or under 1 g per day,
134 were excluded as they were almost certainly erroneous. There were three occasions in which an
135 estimated value for weekly food intake was used, so that we did not lose data for the entire mouse.
136 In the female cohort, food intake for a single PR mouse for week 4 was estimated by averaging
137 the weekly intake of this mouse on all other weeks. This was similar to the approach taken in the
138 male cohort for a single NR mouse during week 2. In the male cohort a PR mouse had its baseline

intake estimated by using the average intake of all the other mice. The average food intake for each mouse on weeks when the IPR group was on NR, and when the IPR group was on PR, were compared between groups. A mixed model ANOVA was conducted, with Group as between-groups factor and Type of Week (IPR on NR diet, or IPR on PR diet) as within-groups factor.

Plasma ghrelin was analyzed as a factorial ANOVA, with Group and Condition as factors. Due to the number of samples able to be run on a single ELISA plate, 3 female mice (1 NR, 1 PR, 1 IPR, all from the "fed" condition) were not included. One mouse (male NR fasted) was two standard deviations above the group mean and was therefore considered an outlier and removed from the analysis.

3. Results

3.1 Body weight

In males, body weight, as expected, increased over time as evidenced by a main effect of Time (Fig 1B; $F_{8, 312} = 357.159$, $p < 0.001$). There was also a main effect of Group ($F_{2, 39} = 4.425$, $p = 0.019$), as well as a Time x Group interaction ($F_{16, 312} = 22.177$, $p < 0.001$). Post-hoc tests on the interaction revealed that by week 6, IPR mice were significantly heavier than PR mice. By week 7, NR mice were also heavier than PR mice, and both groups were heavier than PR mice also at week 8. At no point did IPR mice and NR mice differ in body weight.

In females, body weight also increased over time (Fig. 1C; $F_{8, 264} = 113.22$, $p < 0.001$). There were no differences between the groups ($F_{2, 33} = 0.047$, $p = 0.954$), nor a Group x Time interaction.

3.2 Body composition

In males, fat mass increased between baseline and after eight weeks on experimental diets (Fig 2A; main effect of Time, $F_{1, 39} = 305.706$, $p < 0.001$). There was also a significant main effect of Group ($F_{2, 39} = 22.91$, $p < 0.001$), and a Group x Time interaction ($F_{2, 39} = 9.813$, $p < 0.001$). Post-hoc tests on this interaction indicated that although each of the groups had a higher fat mass at the end of the experiment than at the beginning, PR mice had significantly less fat mass at the end than either NR mice or IPR mice; NR and PR mice did not differ from one another (Fig 2E). Analysis of lean mass also showed a significant main effect of Time (Fig 2C; $F_{1, 39} = 50.109$, $p < 0.001$), but no main effect of Group ($F_{2, 39} = 2.178$, $p = 0.2$). The Time x Group interaction was significant ($F_{2, 39} = 8.5$, $p < 0.001$), and post-hoc tests showed that only the NR mice and IPR mice gained lean mass over the eight weeks of the experiment (Fig 2G). At the end of the experiment

IPR mice had a higher lean mass than did PR mice, but PR mice and NR mice did not differ in lean mass.

In females, fat mass increased over time (Fig 2B; $F_{1, 33} = 79.768$, $p < 0.001$) but there was no main effect of Group ($F_{2, 33} = 0.644$, $p = 0.532$) nor a Group x Time interaction (Fig 2F; $_{2, 33} = 1.058$, $p = 0.359$). A similar pattern of results was found for lean mass, such that lean mass was higher at the end of the experiment than at baseline (Fig 2D; $F_{1, 33} = 329.289$ $p < 0.001$), but there were no group differences ($F_{2, 33} = 0.529$, $p = 0.722$) nor Group x Time interaction (Fig 2H; $F_{2, 33} = 1.359$, $p = 0.271$).

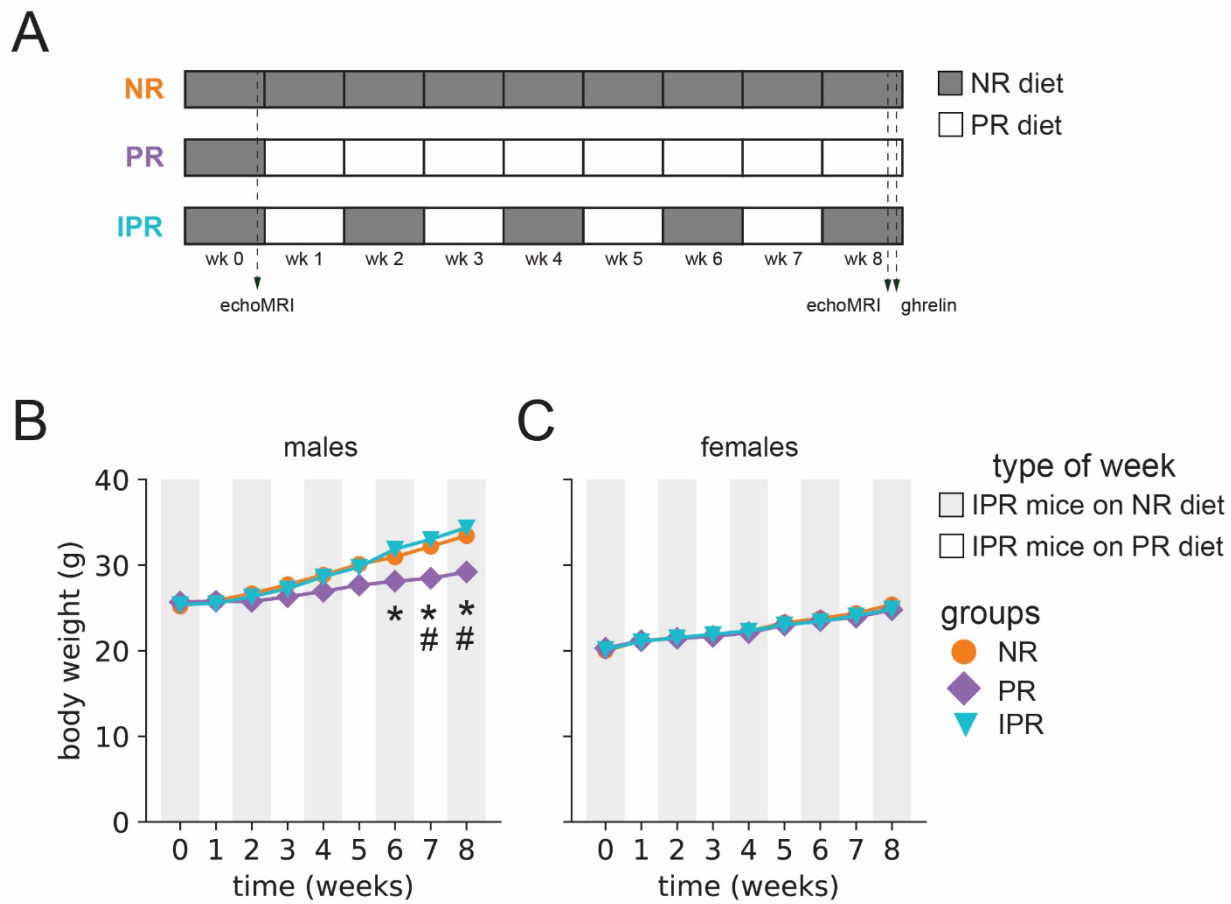

Figure 1: Schematic of experimental groups, and body weight. After a week of baseline measurements while on a normal protein diet (NR), some mice continued eating only this diet, some were given a protein-restricted diet (PR), and others alternated between the two diets (IPR) (A). Over the course of the experiment, body weight increased in male (B) and female (C) mice. Male mice on PR diet weighed less than IPR mice from week 6 and onwards, and less than those NR diet on weeks 7 and 8. Groups did not differ in body weight in female mice. Data are mean +/- SEM. Error bars too small to be visible. * PR differs from IPR, # PR differs from NR.

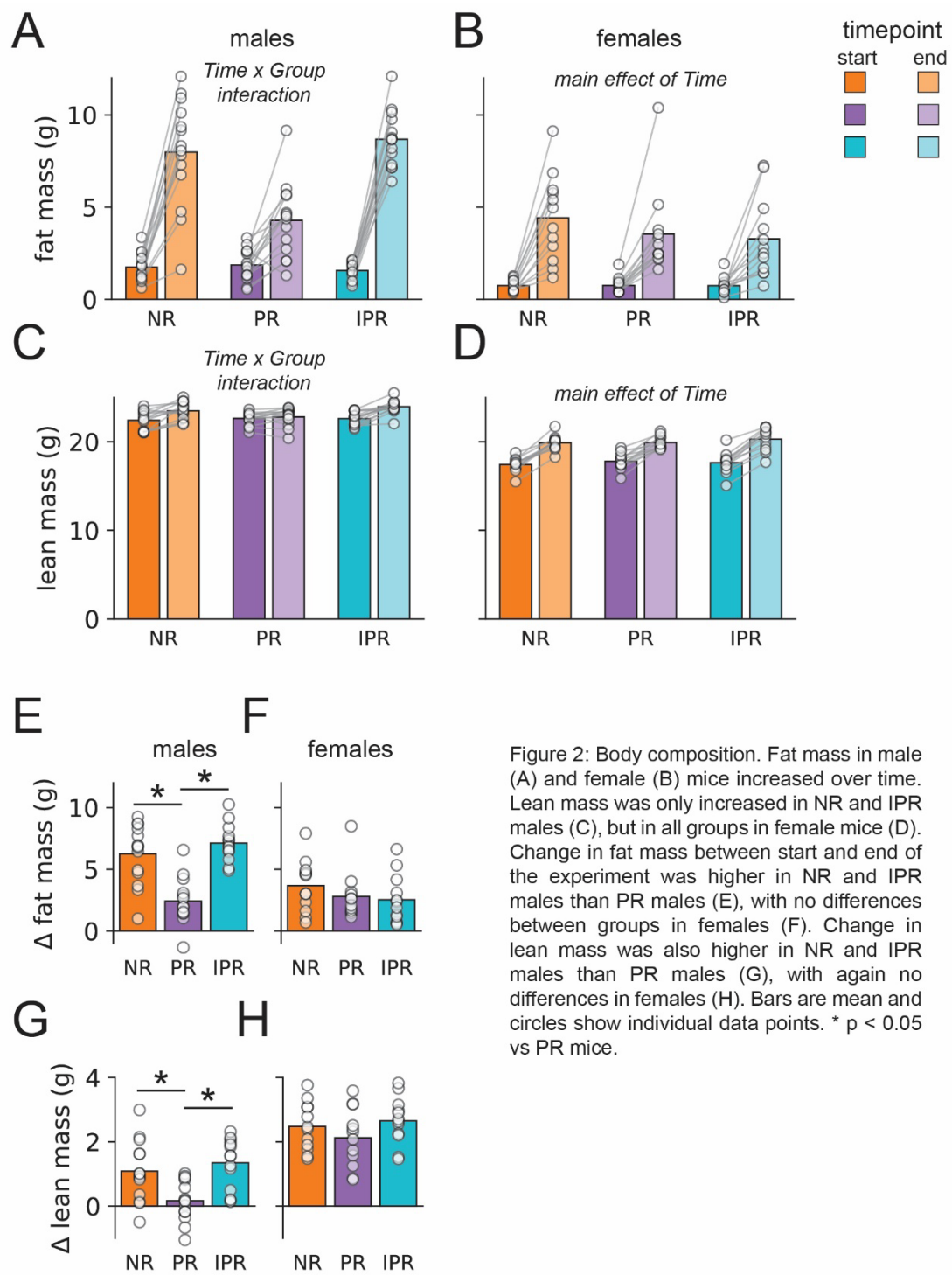

Figure 2: Body composition. Fat mass in male (A) and female (B) mice increased over time. Lean mass was only increased in NR and IPR males (C), but in all groups in female mice (D). Change in fat mass between start and end of the experiment was higher in NR and IPR males than PR males (E), with no differences between groups in females (F). Change in lean mass was also higher in NR and IPR males than PR males (G), with again no differences in females (H). Bars are mean and circles show individual data points. * $p < 0.05$ vs PR mice.

3.3 Food intake

In males, average food intake consumed each week was different across time (Fig 3A; $F_{8, 312}$ = 7.565, $p < 0.001$) and between groups ($F_{2, 39}$ = 27.66, $p < 0.001$), and there was a significant Time x Group interaction ($F_{16, 312}$ = 2.643, $p < 0.001$). Post-hoc testing revealed that NR mice ate less food than did PR mice on weeks 3, 4, 6, and 8, and less than IPR mice did on weeks 6, 7, and 8;

188 IPR mice were eating NR diet on weeks 6 and 8. Furthermore, we compared the average intake
189 of mice during weeks in which the IPR group was on NR diet or PR diets (Fig 3C). Here, we found
190 that intake did not differ by type of week (IPR mice on NR diet vs. PR diet) ($F_{1, 39}$ = 0.004, p =
191 0.947), but there was a significant main effect of Group ($F_{2, 39}$ = 2.272, p < 0.001) and a Type of
192 Week x Group interaction ($F_{2, 39}$ = 4.507, p = 0.017). As expected, neither NR mice nor PR mice
193 ate different amounts of their respective diets depending on whether IPR mice were eating NR or
194 PR diet, but, interestingly, on average food intake also did not differ in IPR mice depending on

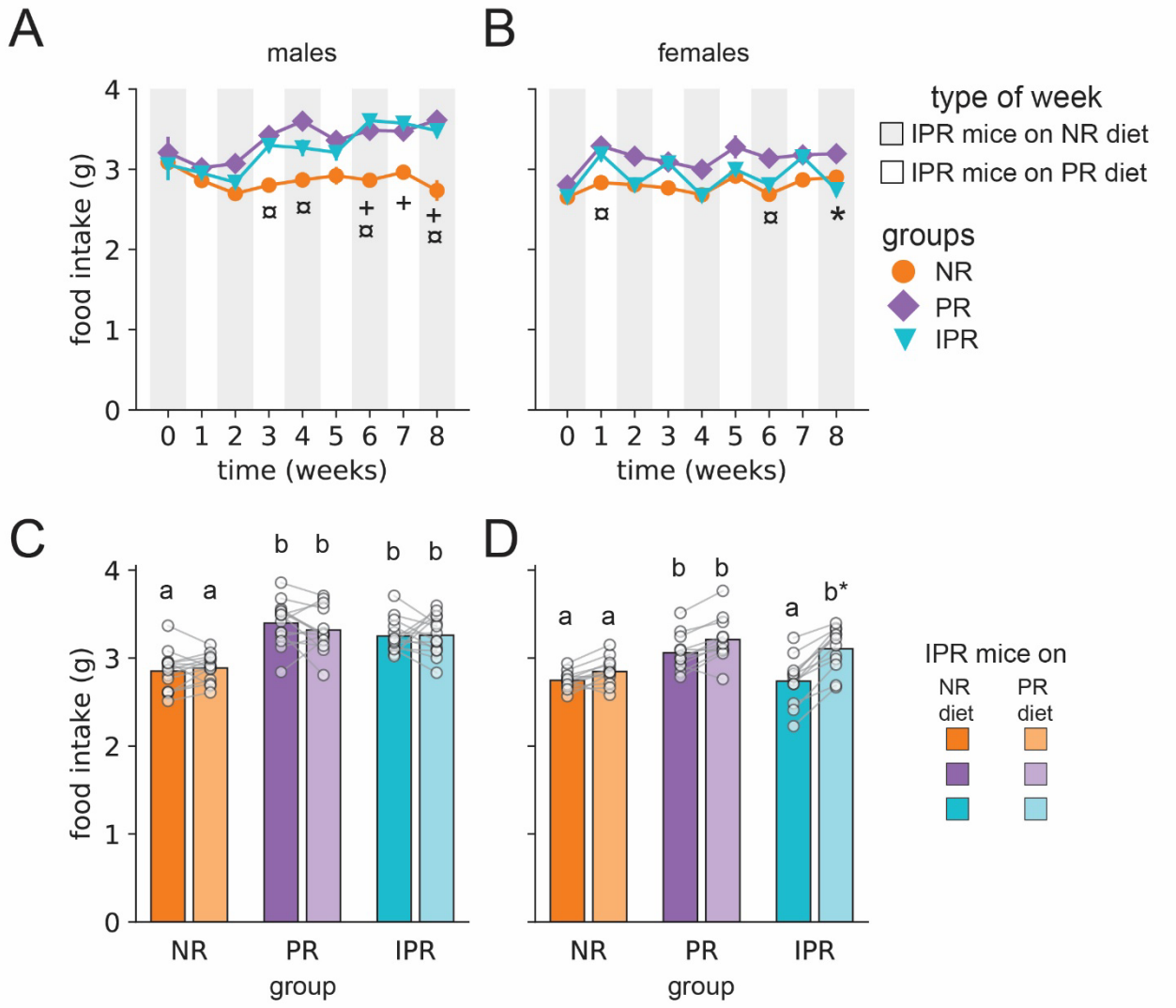

Figure 3: Food intake. Male PR mice ate significantly more than NR mice on weeks 3,4,6 and 8; IPR male mice are more than NR mice on the last three weeks of the experiment (A). In female mice, PR mice ate more than NR on weeks 1 and 6, while IPR ate more than PR on week 8 (B). Average intake on weeks in which IPR was on NR diet or PR diet did not differ in any male groups, but PR and IPR males ate more than NR males regardless of week type (C). In female mice, IPR mice ate more when on PR diet than NR diet; their intake was well-matched to that of the mice on the chronic diets (D). In A and B, data are mean +/- SEM. Error bars are too small to be visible. In C and D, bars are mean and circles show individual data points. ¤ NR differs from PR, * PR differs from IPR, + IPR differs from NR, lower-case letters indicate p < 0.05 (e.g., a is different from b, b* p = 0.05 difference vs NR mice when IPR mice are on PR diet).

which diet they were currently consuming. Regardless of which diet they were eating, IPR mice ate the same amount as PR mice and more than NR mice.

In females, food intake by week had significant main effects of Time (Fig 3B; $F_{8, 264}$ = 15.532, $p < 0.001$) and Group ($F_{2, 33}$ = 8.46, $p < 0.001$), as well as a significant Time x Group interaction ($F_{16, 264}$ = 3.05, $p < 0.001$). Post-hoc tests on the interaction revealed that NR mice and PR mice differed in intakes on weeks 1 and 6, while PR mice and IPR mice differed in intakes on week 8. Average intake on weeks when IPR mice were on PR diet or NR diet similarly had main effects of Type of Week (Fig 3D; $F_{1, 33}$ = 66.354, $p < 0.001$) and Group ($F_{2, 33}$ = 9.506, $p < 0.001$), and a Type of Week x Group interaction ($F_{2, 33}$ = 18.108, $p < 0.001$). Post-hoc tests revealed that intakes of NR mice and PR mice remained stable (as expected), with NR mice eating less than PR mice. IPR mice, on the other hand, ate significantly more when on PR diet than on NR diet. When IPR mice were on NR diet their intake was the same as NR mice and lower than that of PR mice; when IPR mice were on PR diet their intake was the same as PR mice and there was a trend (p=0.053) towards their intake being higher than NR mice.

3.4 Plasma ghrelin

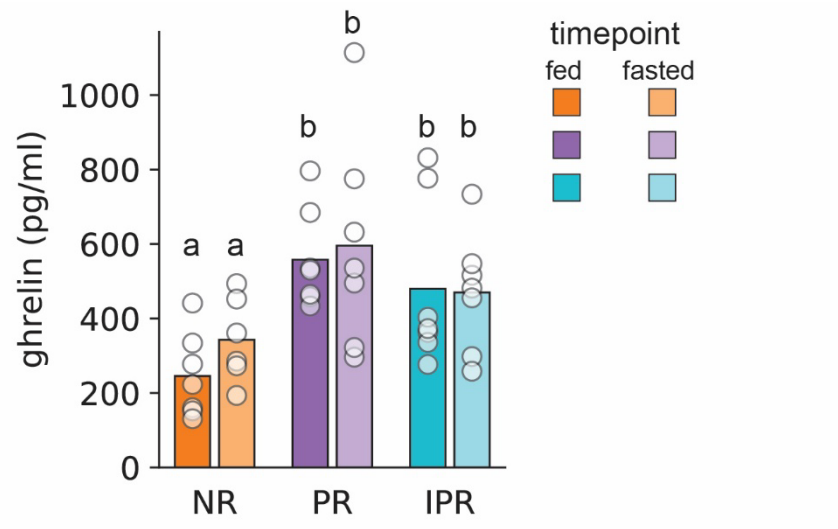

Figure 4: Plasma ghrelin in male mice. Male on PR diet, and IPR mice currently eating NR diet, both had higher plasma ghrelin than did NR mice. Bars are mean and circles show individual data points, lower-case letters indicate $p < 0.05$ (e.g., a is different from b).

Plasma ghrelin levels in a fed and fasted state were analyzed with repeated measures ANOVA. We found a main effect of Group ($F_{2, 35}$ = 8.041, p = 0.001) and no effect of Condition ($F_{1, 35}$ = 0.529, p = 0.472), nor an interaction ($F_{2, 35}$ = 0.286, p = 0.753). Post-hoc tests revealed that both PR mice and IPR mice had higher plasma ghrelin than did NR mice.

In the female mice, many samples (9 out of 34) were out of range for the ELISA. Of these, all but one were from the fed state. We therefore ran an ELISA only on the fasted state, but found no effect of Group ($F_{2, 14}$ = 2.167, p = 0.151).

4. Discussion

Mice experiencing intermittent protein restriction did not show an intermediate phenotype between that of NR and PR mice. In male IPR mice, IPR mice were indistinguishable from NR control mice in terms of body weight and body composition. One reason could be that the average level of protein restriction was less in the IPR group. They spent half the time eating a 5% protein diet, and half the time eating a 20% protein diet, so on average were eating 12.5% protein. This may be sufficient for maintaining growth, although it is below the 13.6% that has been found to support growth and reproductive health in mice (Goettsch, 1960). This is important to consider because low-protein diets (and especially those low in certain amino acids) have been suggested as ways of improving metabolic health (for example, (Fontana et al., 2016; Rang et al., 2021). The present experiment highlights that at least some of these benefits may be contingent on long-term use of the diet or avoiding frequently switching between a low and normal protein diet. Female mice, regardless of group, did not differ in body weight or composition.

Interestingly, one measure in which intermittent protein restriction differed from normal diet was in food intake. Protein restriction leads to higher caloric intake per day; this has been demonstrated many times in male mice (Fontana et al., 2016; Green et al., 2022; Hill et al., 2019; Laeger et al., 2014; Trautman et al., 2023; Volcko & McCutcheon, 2022) and is sometimes observed in female mice (Blais et al., 2018; Volcko & McCutcheon, 2022). In males, we found that the increase in food intake persisted even when the IPR male mice were consuming the control diet. Presumably, this persistent hyperphagia allowed the mice to compensate for a period when a reduced amount of protein was available. Understanding precise parameters underlying this compensatory behavior – e.g., how persistent is the hyperphagia and, if a choice is available, is it directed towards certain foods? – will be important for understanding any consequences for human health and eating behavior. As such, future experiments should address these questions as well as consider which parts of the brain are altered by protein restriction to promote hyperphagia of both low- and normal-protein food sources. Importantly, a recent paper has reviewed the persistence of both behavioral responses and physiological parameters such as a change in neural activity (Soto & Morrison, 2024). The authors argue that behavior and physiology are dictated not solely by current nutritional requirements but also by historical nutritional conditions.

Female mice on intermittent protein restriction did not show persistent hyperphagia, but were able to adapt their intake to whichever diet they were eating at the time. As others have pointed out, sex is an important factor in the effects of protein restriction (Green et al., 2022). It is worth noting that food intake was measured differently in male and female mice: in males, two 24-

263 hour measurement periods (one immediately before and one immediately after IPR changed diets)
264 were averaged to form a mean daily intake per week, whereas in female mice total food consumed
265 in a week was divided by 7 for a weekly average intake. In female mice we did measure 24-hour
266 intake on a few occasions, and correlated the intakes using both methods. The R^2 value (0.72 and
267 0.78, on two separate weeks, $p < 0.001$ on both occasions) demonstrates that using two methods
268 may add some variability, but the relationship is strong enough that we feel confident in the major
269 interpretation of the data: male mice on IPR show persistent high levels of intake regardless of
270 their diet, whereas female mice on IPR do not.

271 Ghrelin levels, surprisingly, did not differ by sampling time point – i.e., "fed" vs. "fasted" –
272 in our experiment. In the female cohort many samples from the "fed" state were below the
273 detection threshold, indicating that condition (fed vs. fasted) did affect ghrelin levels. In the male
274 cohort this was not the case; although this could be a sex difference, it is also likely that the minor
275 difference in timing was important. In females, blood was sampled at the end of the dark phase
276 and at the end of the light phase; in males, blood was sampled at the beginning of the light phase
277 and at the beginning of the dark phase. This is a small difference in clock time but perhaps a very
278 significant one in ghrelin secretion. We chose the time points with the assumption that ghrelin
279 would be high after a short fast and at the beginning of the dark phase when eating normally
280 occurs, and would be lower at the beginning of the light phase after the mice had experienced ad
281 libitum overnight access to their respective diets. We did not find this, but the results of this
282 experiment are nonetheless intriguing. In males there was a main effect of Diet that showed higher
283 plasma ghrelin in PR mice than in NR mice, similar to what is seen in rats (Chaumontet et al.,
284 2018). IPR mice, interestingly, had as high plasma ghrelin as PR, despite blood sampling
285 occurring a week after they had returned to NR diet. Whether or not this potential difference in
286 ghrelin might underlie some of the increased food consumption seen in PR and IPR mice warrants
287 further study.

288 Female mice on protein-restricted diet were remarkably similar to their controls eating a
289 diet with sufficient protein, in all measures but food intake. This is striking when viewed in light of
290 their protein-seeking behavior. Although we did not measure protein preference in this experiment,
291 we have previously shown that female mice have as robust a shift in macronutrient preference as
292 do male mice experiencing protein restriction (Volcko & McCutcheon, 2022). The lack of a change
293 in body weight or composition in female mice on this diet indicates that they are able to obtain
294 enough protein by increasing their intake of protein-restricted diet, while males are not able to do
295 so. One might speculate that this would lead to a greater drive to consume protein in males than
296 females; in a sense, a male mouse can perhaps be seen as experiencing a higher degree of

297 restriction on the same diet, because its protein requirements are higher. And yet, we do not see
298 a commensurate increase in protein appetite. This is intriguing and suggests that the relationship
299 between motivation to consume protein, and protein need, is not entirely linear.
300 In summary, here we add support to earlier studies showing that male and female mice
301 respond differently when challenged with a low-protein diet with respect to body weight, body
302 composition, and food intake. Moreover, male mice completely compensate for repeated periods
303 of low-protein diet exposure by increasing food intake, both while on the PR diet and for at least a
304 week after return to a diet with a normal protein level. A higher level of plasma ghrelin may be at
305 least partially involved in this hyperphagia, although a causal link was not studied here.
306

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311 **Author contributions**
312 **Volcko, K.L**: Conceptualization, Investigation, Writing – Original Draft. **Taghipourbibalan, H**:
313 Investigation. **McCutcheon, J.E**: Conceptualization, Supervision, Writing – Review and Editing.
314 All authors have approved this manuscript.
315
316

Figure legends

Figure 1: Schematic of experimental groups, and body weight. After a week of baseline measurements while on a normal protein diet (NR), some mice continued eating only this diet, some were given a protein-restricted diet (PR), and others alternated between the two diets (IPR) (A). Over the course of the experiment, body weight increased in male (B) and female (C) mice. Male mice on PR diet weighed less than IPR mice from week 6 and onwards, and less than those NR diet on weeks 7 and 8. Groups did not differ in body weight in female mice. Data are mean +/- SEM. Error bars too small to be visible. * PR differs from IPR, # PR differs from NR.

Figure 2: Body composition. Fat mass in male (A) and female (B) mice increased over time. Lean mass was only increased in NR and IPR males (C), but in all groups in female mice (D). Change in fat mass between start and end of the experiment was higher in NR and IPR males than PR males (E), with no differences between groups in females (F). Change in lean mass was also higher in NR and IPR males than PR males (G), with again no differences in females (H). Bars are mean and circles show individual data points. * $p < 0.05$ vs PR mice.

Figure 3: Food intake. Male PR mice ate significantly more than NR mice on weeks 3,4,6 and 8; IPR male mice are more than NR mice on the last three weeks of the experiment (A). In female mice, PR mice ate more than NR on weeks 1 and 6, while IPR ate more than PR on week 8 (B). Average intake on weeks in which IPR was on NR diet or PR diet did not differ in any male groups, but PR and IPR males ate more than NR males regardless of week type (C). In female mice, IPR mice ate more when on PR diet than NR diet; their intake was well-matched to that of the mice on the chronic diets (D). In A and B, data are mean +/- SEM. Error bars are too small to be visible. In C and D, bars are mean and circles show individual data points. ¤ NR differs from PR, * PR differs from IPR, + IPR differs from NR, lower-case letters indicate $p < 0.05$ (e.g., a is different from b, b* $p = 0.05$ difference vs NR mice when IPR mice are on PR diet).

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