Title: Restriction of dietary protein in rats increases progressive-ratio motivation for protein

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Abstract

Low-protein diets can impact food intake and appetite, but it is not known if motivation for food is changed. In the present study, we used an operant behavioral task – the progressive ratio test – to assess whether motivation for different foods was affected when rats were maintained on a protein-restricted diet (PR, 5% protein diet) compared to non-restricted control rats (NR, 18% protein). Rats were tested either with nutritionally-balanced pellets (18.7% protein, Experiment 1) or protein-rich pellets (35% protein, Experiment 2) as reinforcers. Protein restriction increased breakpoint for protein-rich pellets, relative to non-restricted rats, whereas no difference in breakpoint for nutritionally-balanced pellets was observed between groups. When given free access to either nutritionally-balanced pellets or protein-rich pellets, PR and NR rats did not differ in their intake. We also tested whether a previous history of protein restriction might affect present motivation for different types of food, by assessing breakpoint of previously PR animals that were subsequently put on standard maintenance chow (protein-repleted rats, PRep, Experiment 2). PRep rats did not show increased breakpoint relative to their initial encounter with protein-rich pellets while they were protein-restricted. This study demonstrates that restriction of dietary protein induces a selective increased motivation for protein-rich food, a behavior that rapidly disappears once rats are not in need of protein.

Key words: amino acids; protein; diet; motivation; progressive ratio; rat

1. Introduction

The motivation to consume food strongly influences the amount of food consumed. In the context of maintaining homeostasis, increased motivation for food operates to restore energy or nutrient-specific depletion (Lutter & Nestler, 2009). In animal models, food restriction, for example, enhances motivation for highly caloric food (Jewett et al., 1995; Sharma et al., 2012). Similarly, in regards to sodium homeostasis, sodium depletion specifically increases operant responding for salt (Clark & Bernstein, 2006; Krieckhaus & Wolf, 1968; Quartermain et al., 1967) and enhances the motivational value of salt-associated cues (Robinson & Berridge, 2013).

The impact of dietary protein intake on cognitive functions is a subject of growing interest. In humans, maternal protein insufficiency causes offspring to have deficits in learning, memory and operant responding for a food reward (Gould et al., 2018; Grissom & Reyes, 2013). Poorer cognitive functions in several domains (e.g. registration, attention, calculation, orientation, executive function) are reported in adults and older people on low-protein diets (Dickerson et al., 2020; Richard et al., 2018). In rodents, the importance of perinatal protein sufficiency for cognitive development has been demonstrated extensively (Almeida et al., 1996; Levitsky et al., 1975; McGaughy et al., 2014; Rushmore et al., 2021; Tonkiss et al., 1991a; Tonkiss & Galler, 1990; Tonkiss et al., 1991b). Notably, the effects of maternal protein malnutrition on spatial working memory and spatial learning are observed even trans-generationally (i.e. F_2) (Abey et al., 2019). In adult rats, acute depletion of the essential amino acid tryptophan leads to impaired object recognition, increased anxiety and depression-related behavior (Jans et al., 2010). In aging mice, protracted protein deficiency causes learning and memory deficits, which are reversed by essential amino acids administration (Sato et al., 2020). Overall, these studies indicate that cognitive impairments, especially in learning and memory, are strongly linked to protein deficiency. However, there is a lack of research investigating the consequences of protein restriction on motivation for food in rodents.

Our lab and others' have recently demonstrated that rodents maintained on a protein-restricted diet develop a strong preference for protein-containing food, relative to carbohydrate (Chiacchierini et al., 2021; Hill et al., 2019; Murphy et al., 2018; Naneix et al., 2020). Moreover, we recently showed that protein restriction impacts dopamine release (Naneix et al., 2021) and changes the response of ventral tegmental area neurons to the

consumption of protein-containing food (Chiacchierini et al., 2021). What is not yet clear is whether this behavioral adaptation is also associated with changes in the motivation to obtain protein-rich food. Here, protein-restricted (PR) and control rats (non-restricted, NR) were trained to respond for pellets with differing protein content (nutritionally-balanced, 18%; protein-rich, 35%) and tested on a progressive ratio task in order to assess nutrient-specific changes in motivation. Additionally, we assessed whether a history of protein restriction affected motivation for protein-rich and nutritionally-balanced pellets when a nutritionally-balanced maintenance diet was restored.

2. Materials and Methods

2.1. Animals

Adult male Sprague Dawley rats were used for experiments (Experiment 1, n = 15; Experiment 2, n = 15. Charles River, weight range: 325-360 g; mean: 346 g at start of experiments). Rats were housed in pairs in individually ventilated cages (46.2 x 40.3 x 40.4 cm) with bedding material as recommended by NC3R guidelines. Temperature was 21 ± 2 ˚C and humidity was 40-50%, with 12:12 h light/dark cycle (lights on at 07:00 am). Water and food were available ad libitum. Two rats were removed from the study because they did not show any instrumental learning during and after training (see section 2.6 for exclusion criteria). All experiments were covered by the Animals [Scientific Procedures] Act (1986) and carried out under the appropriate license authority (Project License: PFACC16E2).

2.2. Diets

All rats were initially maintained on standard laboratory chow (Teklad Global 18% Protein Rodent Diet, Envigo). A week after arrival, half of the rats were randomly assigned to the PR diet condition (Experiment 1, n=7; Experiment 2, n=7). For these rats, standard chow was switched to a modified AIN-93G diet containing 5% protein from casein (#D151000, Research Diets; (Murphy et al., 2018)). Remaining rats were maintained under standard laboratory chow diet (NR; Experiment 1, n=8; Experiment 2, n=8). Behavioral testing started 1 week after diet manipulation.

F0021 (nutritionally-balanced)	F07589 (casein-rich)
18.7% Protein	35% Protein (Casein)
59.1% Carbohydrate	0.5% L-Methionine
4.7% Fibre	64.5% Fibre
5.6% Fat	
6.5% Ash	
< 10% Moisture	

Table 1 Reinforcers used in the study. Chemical composition of the food pellets used as reinforcers in Experiment 1 (#F0021) and in Experiment 2 (#F07589).

2.3. Food reinforcers

Nutritionally-balanced pellets (F0021, BioServ) or protein-rich pellets (35% casein; F07589, BioServ) (**Table 1**) were used as reinforcers in Experiment 1 and Experiment 2, respectively.

2.4. Testing apparatus

Rats were tested in standard operant chambers (25 x 32 x 25.5 cm, Med Associates) placed inside sound attenuating chambers (1200 x 700 x 700 cm) with inbuilt ventilation fans. Each conditioning chamber was equipped with a house light located on the left wall while on the right wall there was a custom-designed pellet trough (6 x 6.5 x 2 cm; 3D printed using Open Scad 2015.03 and Ultimaker 2+) and a retractable lever (Med Associates), positioned either on the left or on the right of the pellet trough. The pellet trough was connected to a pellet dispenser (Med Associates) via a plastic tube. The position of the lever (right or left side) was counterbalanced between rats. The house light was turned on at the beginning of the session and turned off at the end of it. All behavioral tests were conducted during the light phase of the light/dark cycle, 5 days a week. Apparatus was controlled and data were recorded onto a PC using MED-PC IV software.

2.5. Magazine training

A week after diet manipulation started, rats were familiarized with the behavioral chamber and pellet delivery system through a magazine training session, in which 50 pellets were delivered into the pellet trough, at pseudo-random intervals (mean inter-pellet interval 40 ± 15 s), over a period of 45 minutes. The lever was retracted during the entire duration of the session.

2.6. Fixed ratio training

Twenty-four hours after magazine training, rats were trained on a fixed-ratio (FR) schedule of reinforcement, during which the lever was always extended. First, rats were trained to press the lever on a FR1 schedule, during which each response resulted in the delivery of one pellet. In subsequent sessions, rats progressed to FR2 (one pellet every two lever presses) and FR5 (one pellet every five lever presses) schedules. For each FR schedule, rats performed a daily session for 5 consecutive days. Reinforced responses were followed by a 5-second timeout period, during which lever presses did not result in additional pellet delivery but the number of lever presses was still recorded. Each FR session was terminated following 45 minutes or 100 pellets earned. Rats earning less than 5% of maximum rewards (i.e., 5 pellets) on at least three consecutive FR5 sessions were excluded from the study.

2.7. Progressive ratio testing

Twenty-four hours after the last training session, rats were tested under a progressive ratio 3 (PR3) schedule for 5 consecutive days. In this test, the number of lever presses required to earn the reinforcer increased progressively by 3 after each reinforcer was delivered, starting at 1 (i.e., 1, 4, 7, 10, etc.). The breakpoint was defined as the last ratio completed before responding ceased. Breakpoint is considered an index of motivation (Hodos, 1961). Sessions stopped after 2 hours or if a reinforcer was not earned for more than 30 minutes.

2.8. Free access testing

Twenty-four hours after the last PR3 session, two daily free access tests were conducted. Rats were placed in the behavioral chambers with the house light on and the lever retracted. For 30 min they had free access to 15 g of pellets in the trough and their food consumption was measured.

2.9. Behavioral timeline

In Experiment 1, nutritionally-balanced pellets (see section 2.3) were used as reinforcers. Rats underwent the magazine training, fixed ratio training, progressive ratio testing and free access testing, as described in previous sections and in **Fig. 1A**.

Experiment 2 was performed with the same timeline of Experiment 1 but using protein-rich pellets as reinforcers. In addition, immediately after the last free access test, protein-restricted rats were placed back onto standard chow (protein repleted rats, PRep). After seven days on standard chow, both NR and PRep rats were tested on 5 daily progressive ratio sessions with casein-rich pellets, followed by 2 daily progressive ratio sessions with nutritionally-balanced pellets (**Fig. 1B**).

2.10. Statistical analysis

Number of responses and responses made during time out period were recorded during fixed ratio and progressive ratio sessions. Breakpoints were recorded during progressive ratio sessions. Statistical analysis was performed using GraphPad Prism 7 and SPSS 24. For the number of responses measured on fixed ratio sessions, three-way mixed ANOVA was used, with Diet as a between-subject variable, and Schedule and Session as within-subject variables. For breakpoints during progressive ratio sessions, two-way mixed ANOVA was used with Diet as between-subject variable and Session as within-subject variable. Session duration was also averaged across the five progressive ratio sessions for each animal, and compared between NR and PR rats with the Log-rank test. Pellet intake (free access tests), average breakpoints, post-reinforcement pause (i.e. time from reinforcer delivery to next lever press), and responses during timeout (progressive ratio tests) were averaged for each rat across sessions and compared between diet groups using unpaired t-tests. For summary data, NR and PR groups were obtained by pooling together animals from Experiment 1 and 2; two-way mixed ANOVA was then used, with Diet as between-subject variable and Pellet type as within-subject variable. Significant effects and interactions were followed, if appropriate, with subsequent post hoc tests. All mixed ANOVAs were checked for sphericity of data using Mauchly's Test and, if this was significant, the Huynh-Feldt corrected values were used. Assumptions of homogeneity of variance and normality were satisfied unless otherwise stated. Alpha was set at $p < 0.05$ and all significance tests were two-tailed. The number of animals was based on estimation from preliminary experiments.

2.11. Data and code availability

All data and custom analysis scripts are available: https://doi.org/10.5281/zenodo.5409201 and https://github.com/mccutcheonlab/PRPR/releases/tag/v0.1.

3. Results

3.1. Experiment 1

3.1.1. Protein restriction does not alter the motivation for nutritionally-balanced food

After magazine training, rats were trained to lever press for nutritionally-balanced pellets using FR1, FR2 and FR5 schedules. To ensure a similar level of training in all rats, each FR schedule was performed on five consecutive daily sessions (**Fig. 1A**). Throughout FR training, number of responses increased over the five sessions similarly in both groups (**Fig. 1B**). As such, three-way mixed ANOVA revealed a main effect of Session ($F(4, 52) = 6.42$, $p < 0.0001$), but no effect of Diet ($F(1, 13) = 1.96$, $p = 0.184$) or Schedule X Diet interaction (2, 13) = 1.44, $p = 0.254$). All other main effects and interactions were irrelevant to our hypothesis.

Following training on FR schedules, rats were tested in five daily progressive ratio sessions, in which the number of lever presses required to earn the next reinforcer increased by three after each reinforcer delivery (PR3). We found that, across repeated PR3 sessions, NR and PR rats reached similar breakpoints. Moreover, breakpoint decreased across sessions in NR rats only (**Fig. 1C**). A two-way mixed ANOVA revealed a significant Diet X Session interaction ($F(4, 52) = 6.32$, $p < 0.001$), a main effect of Session ($F(3.1, 40) = 3.58$, $p = 0.021$) but no main effect of Diet ($F(1, 13) = 0.47$, $p = 0.504$). Subsequent multiple comparisons reported a significant decrease in breakpoint in NR rats across sessions (Dunnett's post hoc tests vs. session 1: session 2, $p = 0.004$; session 3, $p = 0.011$; session 4, $p < 0.001$; session 5, $p = 0.008$) but not in PR rats (all Dunnett's > 0.617). Overall, when all five PR sessions were averaged together, the two diet groups did not differ in the motivation to obtain nutritionally-balanced reinforcers ($t(13) = 0.69$, $p = 0.504$) (**Fig. 1D**). We did not find any difference between groups in the number of responses made during the 5-second timeout (NR, 4.15 ± 2.84; PR, 4.94 ± 4.37; $p = 0.680$) and post-reinforcement pause (NR, 21.56 ± 15.77 s; PR, 18.89 ± 11.53 s; $p = 0.719$), indicating similar engagement in lever pressing behavior.

As the length of PR3 sessions also depended on animals' engagement in lever pressing, we looked at the average duration of PR3 sessions as a further measure of motivation, and found that it was similar between NR and PR rats. The median survival rate for NR rats was 59

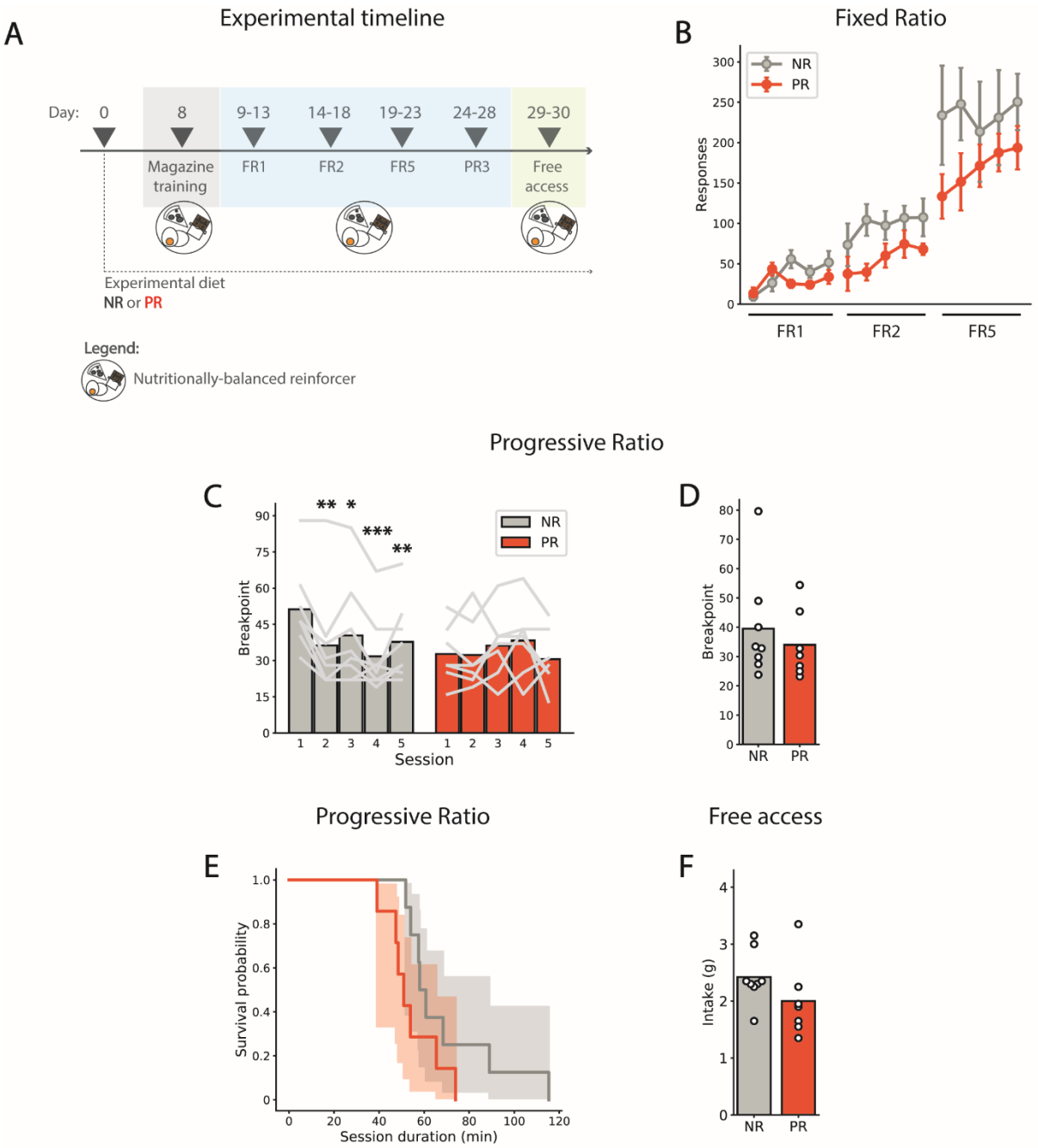

Figure 1 Protein restriction does not alter the motivation for food with nutritionally-balanced content. (A) Timeline of Experiment 1. (B) No difference between non-restricted (NR, grey, n = 8) and protein-restricted rats (PR, red, n = 7) in the number of responses made during fixed-ratio 1 (FR1), FR2 and FR5 sessions (mean ± SEM). (C) PR rats show constant breakpoint across five consecutive progressive ratio 3 (PR3) sessions. NR rats show a decrease in breakpoint across sessions. Bars show mean for each day and grey lines show data from individual rats. (D) No difference between NR and PR rats is observed in the average breakpoint across all days. Bars represent mean and circles represent individual values (rats). (E) Session duration is similar between NR and PR rats. Lines show survival curves for average session duration for all rats and shaded area is confidence interval. (F) NR and PR show similar intake of nutritionally-balanced pellets during free access. Bars represent mean and circles represent individual values (rats). *, **, ***, $p < 0.05$, 0.01, 0.001 vs. Session 1 (Dunnett's post hoc test).

minutes and for PR rats it was 51 minutes (**Fig. 1E**). These survival curves were compared using a Log-rank test, which revealed no difference ($p = 0.101$), further supporting a similar motivation in the two diet groups to work for the pellets.

Following the five PR3 sessions, rats underwent two consecutive daily sessions of free access to the reinforcers (**Fig. 1F**). Pellet consumption across the two sessions was averaged for each rat. Unpaired t-test revealed no difference in the amount of reinforcers consumed between NR and PR rats ($t(13) = 1.4$, $p = 0.177$), indicating that protein restriction does not alter the intake of freely available nutritionally-balanced food.

3.2. Experiment 2

3.2.1. Protein restriction increases the motivation for protein-rich food

The second experiment was performed in a different cohort of rats, to investigate the effects of protein restriction on motivation specifically towards protein. Behavioral procedures were similar as in Experiment 1 but, instead of nutritionally-balanced pellets, protein-rich pellets were used (**Fig. 2A**). During training on FR schedules, PR rats displayed an increased number of lever presses, compared to NR rats (**Fig. 2B**). A three-way mixed ANOVA revealed a main effect of Diet ($F(1, 13) = 6.61$, $p = 0.023$) and a significant Schedule X Diet interaction ($F(1, 13) = 5.76$, $p = 0.032$).

On progressive ratio (PR3) sessions, PR rats reached a higher breakpoint, relative to NR rats. (**Fig. 2C**). A two-way repeated measures ANOVA revealed a main effect of Diet ($F(1, 13) = 26.9$, $p < 0.001$) and of Session ($F(2.34, 30.4) = 3.78$, $p = 0.028$), but no significant interaction ($F(4, 52) = 1.37$, $p = 0.257$). The average breakpoint across sessions confirmed that PR rats were more motivated for protein than NR rats ($t(13) = 5.19$, $p < 0.001$) (**Fig. 2D**). This increased breakpoint was also reflected in a higher survival rate of PR rats when the duration of progressive ratio sessions was analyzed (**Fig. 2E**). As such, the median survival rate of NR rats was 71 minutes, while for PR rats was 82 minutes. Comparison of survival curves revealed a significant difference (Log-rank test, $p = 0.023$).

Analysis of the number of responses made during timeout and the length of post reinforcement pauses identified no significant differences between diet groups (Timeout responses: NR, 6.8 ± 3.72; PR, 13.89 ± 11.51; $p = 0.122$; Post-reinforcement pause: NR, 16.17

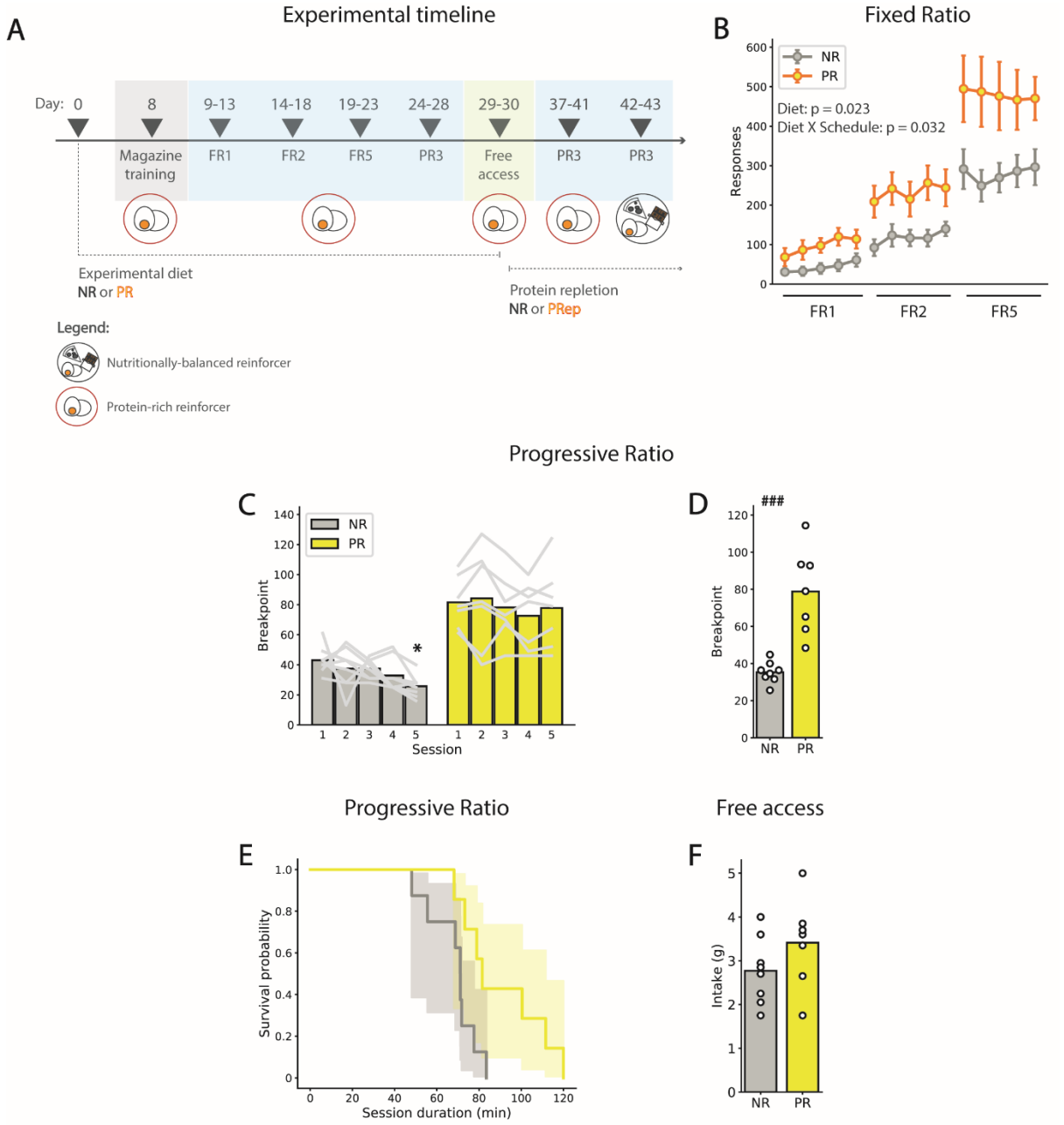

Figure 2 Protein restriction increases the motivation for protein-rich food. (A) Timeline of Experiment 2. (B) During FR sessions, protein-restricted (PR) rats show increased number of responses, compared to non-restricted (NR) rats (mean ± SEM). (C-D) During PR3 sessions, PR rats show elevated breakpoint, relative to NR rats. Bars show mean and grey lines (C) and circles (D) show data from individual rats. (*, $p < 0.05$ vs. Session 1, Dunnett's post hoc test; ###, $p < 0.001$ vs. NR, unpaired t-test). (E) Progressive ratio session duration is longer in PR rats, compared to NR. Lines show survival curves for average session duration for all rats and shaded area is confidence interval. (F) During free access sessions, no difference between diet groups in intake is observed.

± 9.70 s; PR, 13.73 ± 2.76 s; p = 0.532). Interestingly, when rats were given free access to protein-rich pellets for 30 minutes, no difference in intake between diet groups was observed (unpaired t-test: t(13) = 1.40, p = 0.184) (**Fig. 2F**).

3.2.2. Protein repletion rapidly abolishes the increased motivation for protein-rich food

Following the free access test, PR rats were switched back to regular maintenance chow (protein-repleted rats, PRep, **Fig. 2A**). After a week, both NR and PRep rats were tested again on PR3 schedule for protein-rich pellets, for five daily sessions. This allowed motivation for protein-rich food to be assessed in rats with a history of protein restriction, but after protein need state was abolished. We found that NR and PRep rats reached a similar breakpoint, which decreased across sessions (**Fig. 3**) As such, two-way repeated measures ANOVA revealed a main effect of Session ($F(4, 52) = 15.3$, $p < 0.001$), but no effect of Diet ($F(1, 13) = 2.88$, $p = 0.114$) and no interaction ($F(4, 52) = 1.12$, $p = 0.359$). Consistently, the duration of the session was now similar between NR and PRep rats (NR, 70 ± 20 min; PR, 74 ± 24 min; $p = 0.722$). Interestingly, an increase in breakpoint was observed in both diet groups (NR and PRep) when protein-rich reinforcers were replaced by nutritionally-balanced reinforcers (**Fig. 3, shaded columns**). A two-way repeated measures ANOVA revealed a main effect of Session ($F(3.02, 39.2) = 13.6$, $p < 0.001$), but no effect of Diet ($F(1, 13) = 1.78$, $p = 0.205$) and no significant interaction ($F(6, 78) = 1.19$, $p = 0.321$). Subsequent multiple comparisons indicated a progressive decrease in breakpoint, but the trend reverted to initial breakpoint value when nutritionally-balanced pellets were given (**Fig. 3**) (Dunnett's post-hoc tests vs. Session 1: Session 2, $p = 0.263$; Session 3 to Session 5, all $p_s < 0.006$; Session 6 and 7, $p_s > 0.405$).

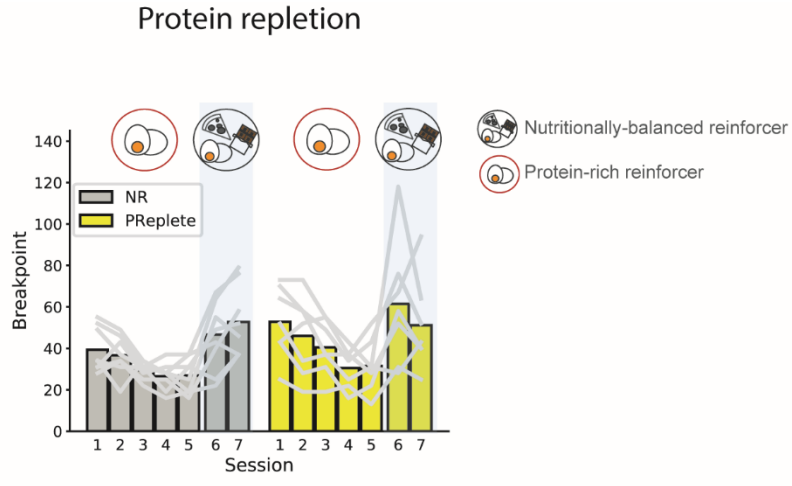

Figure 3 Protein repletion rapidly abolishes the increased motivation for protein food induced by protein need. Following experiment 2, all rats had access to regular maintenance chow for a week (non-restricted, NR and protein-repleted rats, PRep). Both groups then underwent progressive ratio sessions for

protein-rich (Session 1-5) and nutritionally-balanced reinforcers (Session 6 and 7). For protein-rich reinforcers, NR and PRep rats do not differ in breakpoint reached, which decreases across sessions similarly in both groups. However, when protein reinforcers are replaced by nutritionally-balanced reinforcers (Session 6 and 7), both groups show a significant increase in breakpoint, but not different than each other.

3.3. Comparison of progressive-ratio motivation for different reinforcers across all diet conditions

We next analyzed how breakpoint for nutritionally-balanced and protein-rich pellets changed according to the different dietary protein conditions: NR, PR and PRep. Protein status strongly and selectively influenced the motivation for food reinforcers, as shown by a main effect of Diet and a significant Diet X Pellet type interaction (**Fig. 4,** two-way mixed ANOVA: Diet, $F(2, 27) = 4.56$, $p = 0.020$; Diet X Pellet type, $F(2, 27) = 31.0$, $p < 0.001$; no main effect of Pellet type, $p = 0.083$). Further comparisons showed that only current protein restriction led to increased motivation for protein-rich pellets (Tukey's post hoc tests: PR vs. NR, $p < 0.001$; PR vs. PRep, $p < 0.001$; NR vs. PRep, $p = 0.610$). Moreover, protein repletion induced an increase in the motivation for balanced pellets, relative to when rats were protein-restricted (Tukey's post hoc tests: PR vs. PRep, $p = 0.027$; all other $p_s > 0.2$). Interestingly, for PRep rats, there was no significant difference in breakpoint between protein-rich and nutritionally-balanced reinforcers, suggesting that there is not a large difference in incentive value between them (Sidak's post hoc test, $p = 0.054$, **Fig.4**), suggesting that, in PRep rats, protein-rich food has a similar incentive value as regular food, as observed in NR animals.

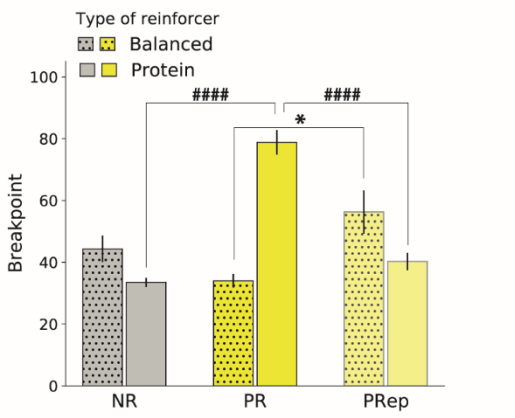

Figure 4 Current and previous protein status strongly and selectively influences motivation for food. Breakpoint for protein-rich reinforcers is elevated in protein-restricted (PR) rats only (yellow bar, center), compared to both non-restricted (NR; grey bar, left) and protein-repleted rats (PRep; pale yellow bar, right). Breakpoint for nutritionally-balanced reinforcers is elevated in PRep rats, relative to PR rats. No difference in breakpoint for nutritionally-balanced reinforcers is observed in NR vs. PR and NR vs PRep (dotted bars). For this summary analysis, NR and PR groups are obtained by pooling together animals from Experiment 1 and 2. Bars show mean ± SEM. *, $p < 0.05$ ####, $p < 0.001$

4. Discussion

The effect of protein restriction on progressive ratio motivation towards food has not yet been determined and this was the main goal of the current study. We found that protein restriction increased the motivation to earn protein-rich food, but not food in general, indicating that protein restriction-induced changes in motivation are selective for protein-rich food. Moreover, restoring protein levels resulted in the rapid abolition of elevated motivation for protein-rich food. Interestingly, despite there being an increased motivation for protein food, when food was freely available its intake was similar between PR and NR rats.

The fulfillment of homeostatic needs such as hunger, thirst or salt appetite is known to drive ingestion-related motivation (Berridge, 2004). As such, food- and water-restricted rodents show increased instrumental responding selectively for the relevant reinforcer (Eiselt et al., 2021; Olarte-Sánchez et al., 2015), demonstrating that depriving rodents of food and water leads to an increase in their incentive value. Similarly, sodium depleted animals are able to perform high-effort sodium-directed activity to restore sodium homeostasis (Quartermain et al., 1967; Schulkin, 1986). Our results suggest that rodents' instrumental behavior also adapts to compensate for protein insufficiency.

In Experiment 1, nutritionally-balanced pellets were used as food reinforcers. During training under FR1, FR2 and FR5 schedules of reinforcement, NR and PR rats made a similar number of lever presses. Conversely, when protein-rich reinforcers were used (Experiment 2), PR rats made an increased number of lever presses already during training sessions. Therefore, the number of responses made during training was predictive of the performance during progressive ratio sessions. Although FR1 and FR2 are low effort schedules of reinforcement and are typically considered a measure of consummatory behavior rather than motivation (Arnold & Roberts, 1997), our data are consistent with other studies reporting a consistency between fixed ratio and progressive ratio measures of reward's motivational properties (Fotio et al., 2021; Velázquez-Sánchez et al., 2014). As regards FR5, it has been proposed as a moderate-effort schedule measuring both intake and motivation (Vendruscolo et al., 2010), therefore the consistency found here between FR5 and PR3 is in support of this idea.

Stable performance on progressive ratio schedule is believed to require at least three sessions (Depoortere et al., 1993; Roberts et al., 1989). We performed five daily progressive ratio sessions and found that, while PR rats show a stable performance, NR rats showed a decrease

in breakpoint across sessions, in both experiments. This decrease in the motivation to obtain protein-rich reinforcers is similar to what happens with calorie-free reinforcers (Beeler et al., 2012). Thus**,** it may be that, with experience, NR rats devalue protein-rich reinforcers due to the lack of other macronutrients in a similar way as rodents do when presented with reinforcers that do not provide nutritional benefit to the organism.

When NR and PR rats were given nutritionally-balanced pellets in the food trough and were free to eat them for 30 minutes (Experiment 1), no difference between groups in total intake was observed. This result is in contrast with previous studies reporting increased food intake as a consequence of moderate protein restriction (Du et al., 2000; Morrison et al., 2012; White et al., 2000), which can be interpreted as a compensatory mechanism to make up for the lack of protein (Hill & Morrison, 2019; Simpson & Raubenheimer, 2005). Surprisingly, even when protein-rich reinforcers were used (Experiment 2), PR rats did not show increased intake during free access, despite an increased breakpoint during the progressive ratio task. This result might prove to be counterintuitive, especially in light of previous data from our lab (Chiacchierini et al., 2021; Murphy et al., 2018) and others (Chaumontet et al., 2018; Hill et al., 2019), showing an increased intake of protein-rich food, relative to carbohydrate, in PR rats when given the choice between the two nutrients. However, in the mentioned studies, protein and carbohydrate-rich food were simultaneously available, which may have resulted in a negative contrast effect (Mitchell & Flaherty, 1998) such as the value of carbohydrate, relative to protein, was decreased as a function of the comparison, leading to increased protein consumption. Conversely, in the present study, rats have free access to a single option (protein-rich food), therefore the lack of comparison with carbohydrate might have resulted in no increased intake in PR rats. In line with this idea, the lack of increased intake of protein in PR rats in the absence of a choice between nutrients has been previously reported by our lab during conditioning and forced-choice sessions, when only one nutrient-rich solution was available (Chiacchierini et al., 2021; Murphy et al., 2018). Another possibility to explain the discrepancy between instrumental responding and free access results in Experiment 2 is that protein restriction had the effect of making rats less sensitive to the cost associated with the protein reinforcers, thereby elevating the threshold at which rats can sustainably exert effort. In behavioral economics, this effect is known as “inelastic” demand (Hursh & Silberberg, 2008). It can finally be hypothesized that rats, over the 30-minute free access test, might have

eaten until a maximum and stopped due to satiety, a mechanism that did not seem to be affected by protein restriction.

An important limitation of this study is the inclusion of only a single degree of protein restriction. It is notable, in fact, that different extents of protein restriction leads to different feeding behaviors in rodents, with moderately low-protein diets (between 5 and 10% protein) inducing hyperphagia (Morrison et al., 2007; White et al., 2000), while < 5% protein diets dramatically decrease food intake (Du et al., 2000; Wu et al., 2021; Zapata et al., 2019) - an effect that has been linked to reduced signaling in the hypothalamic hunger-related pathway (Wu et al., 2021). Therefore, further research should be undertaken to investigate the effects of different degrees of protein restriction on food-related motivation.

We have demonstrated for the first time the direct consequences of protein restriction in adult rats on the motivation for different types of food. The next step would be to use this behavioral assay to gain insight into the central mechanisms underlying the increased motivation for protein-rich food induced by protein need state. Work from our group has recently demonstrated an elevated ventral tegmental area neural activity in PR rats consuming a protein-rich solution, relative to carbohydrate (Chiacchierini et al., 2021). In addition, others have reported increased c-Fos protein expression in the nucleus accumbens of PR rats after consuming a high-protein meal, compared to balanced-protein and low-protein meals (Chaumontet et al., 2018). Given the role of mesolimbic dopamine pathway in both the acute effects and learned properties of food rewards (Martel & Fantino, 1996; Tobler et al., 2005) and the involvement of this pathway in homeostatic feeding (Branch et al., 2013; Cone et al., 2014; Sharma et al., 2012), it is likely that changes in motivation induced by protein need are encoded by changes in mesolimbic dopamine. Accordingly, we also recently showed that protein restriction by itself induced specific changes of dopamine release in the nucleus accumbens, but not in the dorsal striatum (Naneix et al., 2021). Neuromodulators such as serotonin have also been shown to be influenced by dietary amino acids content (Markus, 2008). In light of the role of serotonin in the adaptive preference for protein food in flies (Vargas et al., 2010) and the involvement of serotonin transmission in the nucleus accumbens in the regulation of food-directed progressive ratio motivation (Pratt et al., 2012), it is possible that this neurotransmitter is involved in the motivation for protein observed in our PR rats. Finally, humoral signals such as fibroblast growth factor 21 (FGF21) have also been implicated in the response to dietary protein restriction. In particular, FGF21 is increased

in both humans and rodents maintained on a protein-restricted diet (Laeger et al., 2014), and FGF21 signaling in the brain is necessary for the metabolic and behavioral adaptations to protein restriction (Hill et al., 2019). A possibility is that FGF21 interacts with brain pathways responsible for modulating adaptive effort-related behavior in response to protein restriction.

Over the past century, the study of macronutrients' effect on body composition, weight control and on the development of obesity has highlighted the role of carbohydrate and fat in the diet. More recently, it has been proposed that exaggerated consumption of fat and sugar is a compensatory response to the reduction of absolute protein content in the diet, as animals would ingest food for reaching a protein target (Raubenheimer & Simpson, 2019; Simpson & Raubenheimer, 2005). Consistent with this, reports from rodent and human work have shown that protein intake is prioritized over fat and carbohydrate intake in the face of changes in diet composition, resulting in overconsumption of calories when diets are low in protein . In contrast to other studies demonstrating increased food intake in rodents on low-protein diets (Hill et al., 2019; Laeger et al., 2016) , in the present work we did not observe an increase in nutritionally-balanced pellets in response to protein restriction. However, while in the above-mentioned studies food intake was registered daily, in the present work we measured consumption over 30 minutes of free access test.

Given the importance of dietary protein content in the control of food intake, and in light of the deleterious effects caused by aninadequate protein diet on neurodevelopment and cognitive functions (Gould et al., 2018; Grissom & Reyes, 2013), a better understanding of the impact of low protein diet on food-related behaviors and brain regions involved may help to address both health and disease conditions.

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