

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

2

3 **Authors:** Giulia Chiacchierini (1, 2), Fabien Naneix (3), John Apergis-Schoute (1, 4), James E.  
4 McCutcheon (1, 5)

5

6 **Affiliations:**

7 (1) Dept. of Neuroscience, Psychology & Behaviour, University of Leicester, University Road, Leicester,  
8 LE1 9HN, United Kingdom.

9 (2) Genetics of Cognition laboratory, Neuroscience area, Istituto Italiano di Tecnologia, Genova, Italy

10 (3) Rowett Institute, University of Aberdeen, AB25 2ZD, United Kingdom

11 (4) Department of Biological and Experimental Psychology, Queen Mary University of London, London  
12 E1 4NS, United Kingdom

13 (5) Dept. of Psychology, UiT The Arctic University of Norway, Huginbakken 32, 9037 Tromsø, Norway.

14

15 **Corresponding author:** Giulia.Chiacchierini@iit.it

16

17 **Number of pages:** 21

18 **Number of figures:** 4

19 **Number of words:** 7393

20

21 **Conflict of Interest statement:** The authors declare no competing financial interests.

22

23 **Acknowledgements:** The authors acknowledge the help and support from the staff of the Division of  
24 Biomedical Services, Preclinical Research Facility, University of Leicester, for technical support and the  
25 care of experimental animals as well as colleagues in the Department of Neuroscience, Psychology  
26 and Behaviour at the University of Leicester for their academic contribution. This work was funded by  
27 the Biotechnology and Biological Sciences Research Council [grant #BB/M007391/1 to J.E.M.], the  
28 European Commission [grant #GA 631404 to J.E.M.], The Leverhulme Trust [grant #RPG-2017-417 to  
29 J.E.M. and J.A-S.], and Tromsø Research Foundation [grant #19-SG-JMcC to J.E.M.).

30

31 **Author Contributions:** Conceptualization, G.C., J.E.M., F.N., J.A.S.; Formal analysis, G.C. and J.E.M.;  
32 Investigation, G.C. and J.E.M.; Writing, original draft, G.C. and J.E.M.; Review and editing, G.C.,  
33 J.E.M., F.N. and J.A.S.; Funding acquisition, J.E.M. and J.A.S.

34 **Abstract**

35 Low-protein diets can impact food intake and appetite, but it is not known if motivation for  
36 food is changed. In the present study, we used an operant behavioral task – the progressive  
37 ratio test – to assess whether motivation for different foods was affected when rats were  
38 maintained on a protein-restricted diet (PR, 5% protein diet) compared to non-restricted  
39 control rats (NR, 18% protein). Rats were tested either with nutritionally-balanced pellets  
40 (18.7% protein, Experiment 1) or protein-rich pellets (35% protein, Experiment 2) as  
41 reinforcers. Protein restriction increased breakpoint for protein-rich pellets, relative to non-  
42 restricted rats, whereas no difference in breakpoint for nutritionally-balanced pellets was  
43 observed between groups. When given free access to either nutritionally-balanced pellets or  
44 protein-rich pellets, PR and NR rats did not differ in their intake. We also tested whether a  
45 previous history of protein restriction might affect present motivation for different types of  
46 food, by assessing breakpoint of previously PR animals that were subsequently put on  
47 standard maintenance chow (protein-repleted rats, PRep, Experiment 2). PRep rats did not  
48 show increased breakpoint relative to their initial encounter with protein-rich pellets while  
49 they were protein-restricted. This study demonstrates that restriction of dietary protein  
50 induces a selective increased motivation for protein-rich food, a behavior that rapidly  
51 disappears once rats are not in need of protein.

52

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

## 54 **1. Introduction**

55 The motivation to consume food strongly influences the amount of food consumed. In the  
56 context of maintaining homeostasis, increased motivation for food operates to restore energy  
57 or nutrient-specific depletion (Lutter & Nestler, 2009). In animal models, food restriction, for  
58 example, enhances motivation for highly caloric food (Jewett et al., 1995; Sharma et al., 2012).  
59 Similarly, in regards to sodium homeostasis, sodium depletion specifically increases operant  
60 responding for salt (Clark & Bernstein, 2006; Kriekhaus & Wolf, 1968; Quartermain et al.,  
61 1967) and enhances the motivational value of salt-associated cues (Robinson & Berridge,  
62 2013).

63 The impact of dietary protein intake on cognitive functions is a subject of growing interest. In  
64 humans, maternal protein insufficiency causes offspring to have deficits in learning, memory  
65 and operant responding for a food reward (Gould et al., 2018; Grissom & Reyes, 2013). Poorer  
66 cognitive functions in several domains (e.g. registration, attention, calculation, orientation,  
67 executive function) are reported in adults and older people on low-protein diets (Dickerson  
68 et al., 2020; Richard et al., 2018). In rodents, the importance of perinatal protein sufficiency  
69 for cognitive development has been demonstrated extensively (Almeida et al., 1996; Levitsky  
70 et al., 1975; McGaughy et al., 2014; Rushmore et al., 2021; Tonkiss et al., 1991a; Tonkiss &  
71 Galler, 1990; Tonkiss et al., 1991b). Notably, the effects of maternal protein malnutrition on  
72 spatial working memory and spatial learning are observed even trans-generationally (i.e. F<sub>2</sub>)  
73 (Abey et al., 2019). In adult rats, acute depletion of the essential amino acid tryptophan leads  
74 to impaired object recognition, increased anxiety and depression-related behavior (Jans et  
75 al., 2010). In aging mice, protracted protein deficiency causes learning and memory deficits,  
76 which are reversed by essential amino acids administration (Sato et al., 2020). Overall, these  
77 studies indicate that cognitive impairments, especially in learning and memory, are strongly  
78 linked to protein deficiency. However, there is a lack of research investigating the  
79 consequences of protein restriction on motivation for food in rodents.

80 Our lab and others' have recently demonstrated that rodents maintained on a protein-  
81 restricted diet develop a strong preference for protein-containing food, relative to  
82 carbohydrate (Chiacchierini et al., 2021; Hill et al., 2019; Murphy et al., 2018; Naneix et al.,  
83 2020). Moreover, we recently showed that protein restriction impacts dopamine release  
84 (Naneix et al., 2021) and changes the response of ventral tegmental area neurons to the

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

93

## 94 **2. Materials and Methods**

### 95 *2.1. Animals*

96 Adult male Sprague Dawley rats were used for experiments (Experiment 1, n = 15; Experiment  
97 2, n = 15. Charles River, weight range: 325-360 g; mean: 346 g at start of experiments). Rats  
98 were housed in pairs in individually ventilated cages (46.2 x 40.3 x 40.4 cm) with bedding  
99 material as recommended by NC3R guidelines. Temperature was  $21 \pm 2$  °C and humidity was  
100 40-50%, with 12:12 h light/dark cycle (lights on at 07:00 am). Water and food were available  
101 ad libitum. Two rats were removed from the study because they did not show any  
102 instrumental learning during and after training (see section 2.6 for exclusion criteria). All  
103 experiments were covered by the Animals [Scientific Procedures] Act (1986) and carried out  
104 under the appropriate license authority (Project License: PFACC16E2).

105

### 106 *2.2. Diets*

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

114

115

<b>F0021 (nutritionally-balanced)</b>	<b>F07589 (casein-rich)</b>
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).

116 *2.3. Food reinforcers*

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

119

120 *2.4. Testing apparatus*

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

132

133 *2.5. Magazine training*

134 A week after diet manipulation started, rats were familiarized with the behavioral chamber  
135 and pellet delivery system through a magazine training session, in which 50 pellets were  
136 delivered into the pellet trough, at pseudo-random intervals (mean inter-pellet interval  $40 \pm$   
137  $15$  s), over a period of 45 minutes. The lever was retracted during the entire duration of the  
138 session.

139 *2.6. Fixed ratio training*

140 Twenty-four hours after magazine training, rats were trained on a fixed-ratio (FR) schedule of  
141 reinforcement, during which the lever was always extended. First, rats were trained to press  
142 the lever on a FR1 schedule, during which each response resulted in the delivery of one pellet.  
143 In subsequent sessions, rats progressed to FR2 (one pellet every two lever presses) and FR5  
144 (one pellet every five lever presses) schedules. For each FR schedule, rats performed a daily  
145 session for 5 consecutive days. Reinforced responses were followed by a 5-second timeout  
146 period, during which lever presses did not result in additional pellet delivery but the number  
147 of lever presses was still recorded. Each FR session was terminated following 45 minutes or  
148 100 pellets earned. Rats earning less than 5% of maximum rewards (i.e., 5 pellets) on at least  
149 three consecutive FR5 sessions were excluded from the study.

150

151 *2.7. Progressive ratio testing*

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

158

159 *2.8. Free access testing*

160 Twenty-four hours after the last PR3 session, two daily free access tests were conducted. Rats  
161 were placed in the behavioral chambers with the house light on and the lever retracted. For  
162 30 min they had free access to 15 g of pellets in the trough and their food consumption was  
163 measured.

164

165 *2.9. Behavioral timeline*

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

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

175

## 176 *2.10. Statistical analysis*

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

197

## 198 *2.11. Data and code availability*

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

201

### 202 **3. Results**

#### 203 *3.1. Experiment 1*

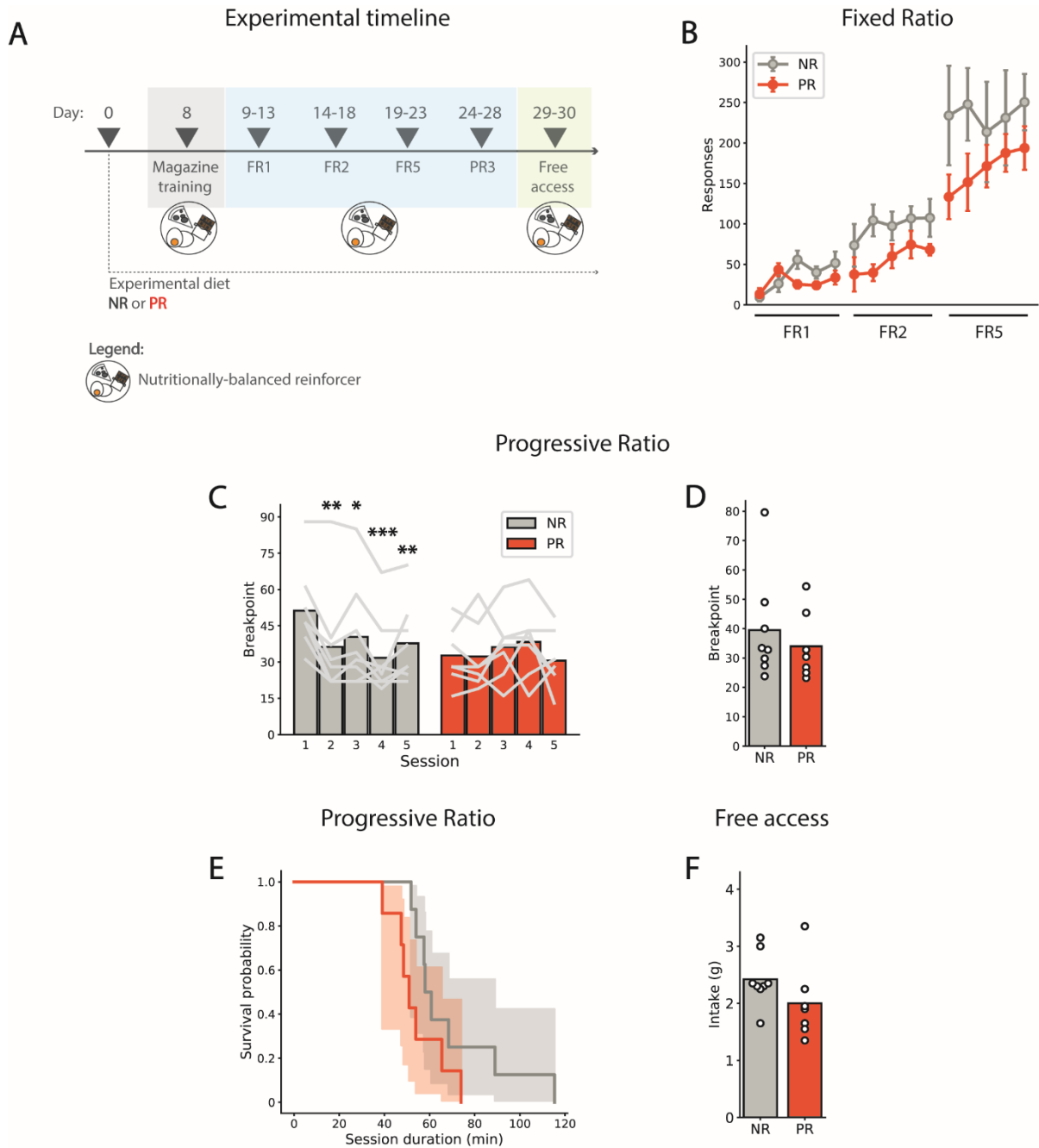
##### 204 *3.1.1. Protein restriction does not alter the motivation for nutritionally-balanced food*

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

212 Following training on FR schedules, rats were tested in five daily progressive ratio sessions, in  
213 which the number of lever presses required to earn the next reinforcer increased by three  
214 after each reinforcer delivery (PR3). We found that, across repeated PR3 sessions, NR and PR  
215 rats reached similar breakpoints. Moreover, breakpoint decreased across sessions in NR rats  
216 only (**Fig. 1C**). A two-way mixed ANOVA revealed a significant Diet X Session interaction ( $F(4,$   
217  $52) = 6.32, p < 0.001$ ), a main effect of Session ( $F(3.1, 40) = 3.58, p = 0.021$ ) but no main effect  
218 of Diet ( $F(1, 13) = 0.47, p = 0.504$ ). Subsequent multiple comparisons reported a significant  
219 decrease in breakpoint in NR rats across sessions (Dunnett's post hoc tests vs. session 1:  
220 session 2,  $p = 0.004$ ; session 3,  $p = 0.011$ ; session 4,  $p < 0.001$ ; session 5,  $p = 0.008$ ) but not in  
221 PR rats (all Dunnett's  $> 0.617$ ). Overall, when all five PR sessions were averaged together, the  
222 two diet groups did not differ in the motivation to obtain nutritionally-balanced reinforcers  
223 ( $t(13) = 0.69, p = 0.504$ ) (**Fig. 1D**). We did not find any difference between groups in the  
224 number of responses made during the 5-second timeout (NR,  $4.15 \pm 2.84$ ; PR,  $4.94 \pm 4.37$ ;  $p$   
225  $= 0.680$ ) and post-reinforcement pause (NR,  $21.56 \pm 15.77$  s; PR,  $18.89 \pm 11.53$  s;  $p = 0.719$ ),  
226 indicating similar engagement in lever pressing behavior.

227 As the length of PR3 sessions also depended on animals' engagement in lever pressing, we  
228 looked at the average duration of PR3 sessions as a further measure of motivation, and found  
229 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  $\pm$  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).

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

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

239

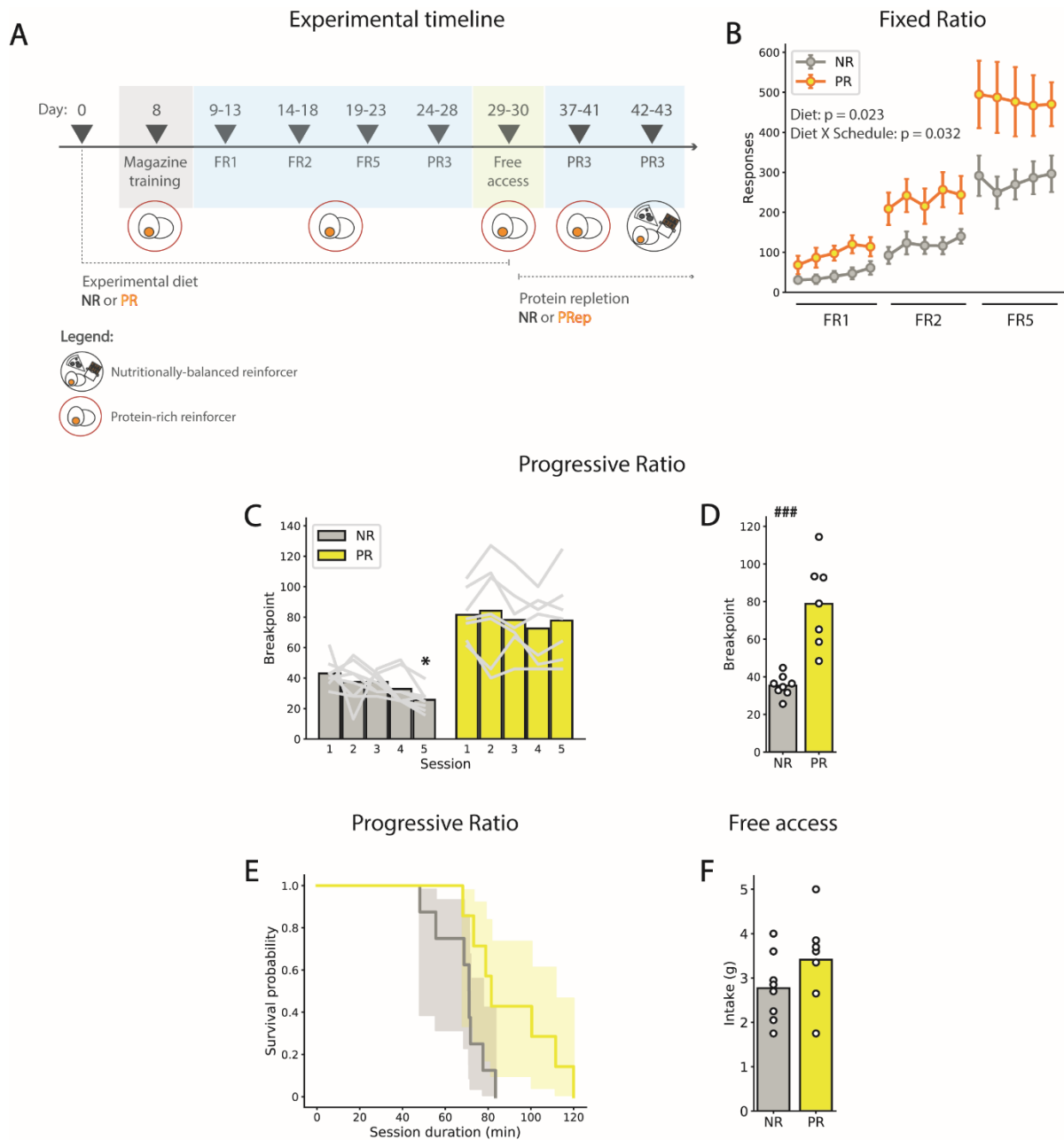
## 240 *3.2. Experiment 2*

### 241 *3.2.1. Protein restriction increases the motivation for protein-rich food*

242 The second experiment was performed in a different cohort of rats, to investigate the effects  
243 of protein restriction on motivation specifically towards protein. Behavioral procedures were  
244 similar as in Experiment 1 but, instead of nutritionally-balanced pellets, protein-rich pellets  
245 were used (**Fig. 2A**). During training on FR schedules, PR rats displayed an increased number  
246 of lever presses, compared to NR rats (**Fig. 2B**). A three-way mixed ANOVA revealed a main  
247 effect of Diet ( $F(1, 13) = 6.61$ ,  $p = 0.023$ ) and a significant Schedule X Diet interaction ( $F(1, 13)$   
248  $= 5.76$ ,  $p = 0.032$ ).

249 On progressive ratio (PR3) sessions, PR rats reached a higher breakpoint, relative to NR rats.  
250 (**Fig. 2C**). A two-way repeated measures ANOVA revealed a main effect of Diet ( $F(1, 13) =$   
251  $26.9$ ,  $p < 0.001$ ) and of Session ( $F(2.34, 30.4) = 3.78$ ,  $p = 0.028$ ), but no significant interaction  
252 ( $F(4, 52) = 1.37$ ,  $p = 0.257$ ). The average breakpoint across sessions confirmed that PR rats  
253 were more motivated for protein than NR rats ( $t(13) = 5.19$ ,  $p < 0.001$ ) (**Fig. 2D**). This increased  
254 breakpoint was also reflected in a higher survival rate of PR rats when the duration of  
255 progressive ratio sessions was analyzed (**Fig. 2E**). As such, the median survival rate of NR rats  
256 was 71 minutes, while for PR rats was 82 minutes. Comparison of survival curves revealed a  
257 significant difference (Log-rank test,  $p = 0.023$ ).

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



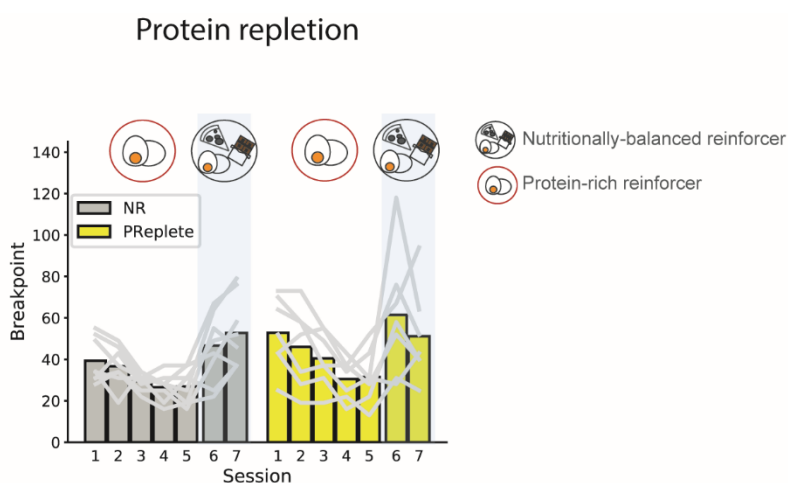
**Figure 2** Protein restriction increases the motivation for protein-rich food. (A) Timeline of Experiment 2. (B) During FR sessions, protein-restricted (PR) rats show increased number of responses, compared to non-restricted (NR) rats (mean  $\pm$  SEM). (C-D) During PR3 sessions, PR rats show elevated 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.

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

264

### 265 3.2.2. Protein repletion rapidly abolishes the increased motivation for protein-rich food

266 Following the free access test, PR rats were switched back to regular maintenance chow  
267 (protein-repleted rats, PRep, **Fig. 2A**). After a week, both NR and PRep rats were tested again  
268 on PR3 schedule for protein-rich pellets, for five daily sessions. This allowed motivation for  
269 protein-rich food to be assessed in rats with a history of protein restriction, but after protein  
270 need state was abolished. We found that NR and PRep rats reached a similar breakpoint,  
271 which decreased across sessions (**Fig. 3**) As such, two-way repeated measures ANOVA  
272 revealed a main effect of Session ( $F(4, 52) = 15.3$ ,  $p < 0.001$ ), but no effect of Diet ( $F(1, 13) =$   
273  $2.88$ ,  $p = 0.114$ ) and no interaction ( $F(4, 52) = 1.12$ ,  $p = 0.359$ ). Consistently, the duration of  
274 the session was now similar between NR and PRep rats (NR,  $70 \pm 20$  min; PR,  $74 \pm 24$  min;  $p =$   
275  $0.722$ ). Interestingly, an increase in breakpoint was observed in both diet groups (NR and  
276 PRep) when protein-rich reinforcers were replaced by nutritionally-balanced reinforcers (**Fig.**  
277 **3, shaded columns**). A two-way repeated measures ANOVA revealed a main effect of Session  
278 ( $F(3.02, 39.2) = 13.6$ ,  $p < 0.001$ ), but no effect of Diet ( $F(1, 13) = 1.78$ ,  $p = 0.205$ ) and no  
279 significant interaction ( $F(6, 78) = 1.19$ ,  $p = 0.321$ ). Subsequent multiple comparisons indicated  
280 a progressive decrease in breakpoint, but the trend reverted to initial breakpoint value when  
281 nutritionally-balanced pellets were given (**Fig. 3**) (Dunnett's post-hoc tests vs. Session 1:  
282 Session 2,  $p = 0.263$ ; Session 3 to Session 5, all  $p_s < 0.006$ ; Session 6 and 7,  $p_s > 0.405$ ).

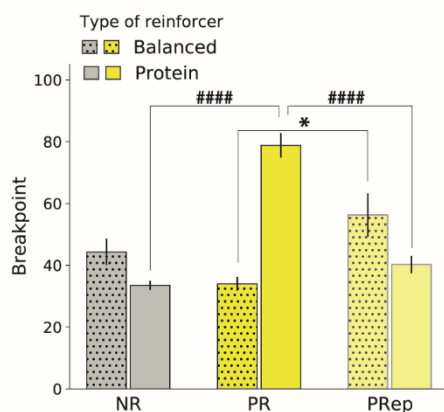


**Figure 3** Protein repletion rapidly abolishes the increased motivation for protein food induced by protein need. Following experiment 2, all rats had access to regular maintenance chow for a week (non-restricted, NR and protein-repleted rats, PRep). Both groups then underwent progressive ratio sessions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

283 *3.3. Comparison of progressive-ratio motivation for different reinforcers across all diet*  
284 *conditions*

285 We next analyzed how breakpoint for nutritionally-balanced and protein-rich pellets changed  
286 according to the different dietary protein conditions: NR, PR and PRep. Protein status strongly  
287 and selectively influenced the motivation for food reinforcers, as shown by a main effect of  
288 Diet and a significant Diet X Pellet type interaction (**Fig. 4**, two-way mixed ANOVA: Diet,  $F(2,$   
289  $27) = 4.56$ ,  $p = 0.020$ ; Diet X Pellet type,  $F(2, 27) = 31.0$ ,  $p < 0.001$ ; no main effect of Pellet  
290 type,  $p = 0.083$ ). Further comparisons showed that only current protein restriction led to  
291 increased motivation for protein-rich pellets (Tukey's post hoc tests: PR vs. NR,  $p < 0.001$ ; PR  
292 vs. PRep,  $p < 0.001$ ; NR vs. PRep,  $p = 0.610$ ). Moreover, protein repletion induced an increase  
293 in the motivation for balanced pellets, relative to when rats were protein-restricted (Tukey's  
294 post hoc tests: PR vs. PRep,  $p = 0.027$ ; all other  $p_s > 0.2$ ). Interestingly, for PRep rats, there  
295 was no significant difference in breakpoint between protein-rich and nutritionally-balanced  
296 reinforcers, suggesting that there is not a large difference in incentive value between them  
297 (Sidak's post hoc test,  $p = 0.054$ , **Fig.4**), suggesting that, in PRep rats, protein-rich food has a  
298 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  $\pm$  SEM. \*,  $p < 0.05$  ####,  $p < 0.001$

#### 299 **4. Discussion**

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

307 The fulfillment of homeostatic needs such as hunger, thirst or salt appetite is known to drive  
308 ingestion-related motivation (Berridge, 2004). As such, food- and water-restricted rodents  
309 show increased instrumental responding selectively for the relevant reinforcer (Eiselt et al.,  
310 2021; Olarte-Sánchez et al., 2015), demonstrating that depriving rodents of food and water  
311 leads to an increase in their incentive value. Similarly, sodium depleted animals are able to  
312 perform high-effort sodium-directed activity to restore sodium homeostasis (Quartermain et  
313 al., 1967; Schulkin, 1986). Our results suggest that rodents' instrumental behavior also adapts  
314 to compensate for protein insufficiency.

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

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

330 in breakpoint across sessions, in both experiments. This decrease in the motivation to obtain  
331 protein-rich reinforcers is similar to what happens with calorie-free reinforcers (Beeler et al.,  
332 2012). Thus, it may be that, with experience, NR rats devalue protein-rich reinforcers due to  
333 the lack of other macronutrients in a similar way as rodents do when presented with  
334 reinforcers that do not provide nutritional benefit to the organism.

335 When NR and PR rats were given nutritionally-balanced pellets in the food trough and were  
336 free to eat them for 30 minutes (Experiment 1), no difference between groups in total intake  
337 was observed. This result is in contrast with previous studies reporting increased food intake  
338 as a consequence of moderate protein restriction (Du et al., 2000; Morrison et al., 2012;  
339 White et al., 2000), which can be interpreted as a compensatory mechanism to make up for  
340 the lack of protein (Hill & Morrison, 2019; Simpson & Raubenheimer, 2005). Surprisingly, even  
341 when protein-rich reinforcers were used (Experiment 2), PR rats did not show increased  
342 intake during free access, despite an increased breakpoint during the progressive ratio task.  
343 This result might prove to be counterintuitive, especially in light of previous data from our lab  
344 (Chiacchierini et al., 2021; Murphy et al., 2018) and others (Chaumontet et al., 2018; Hill et  
345 al., 2019), showing an increased intake of protein-rich food, relative to carbohydrate, in PR  
346 rats when given the choice between the two nutrients. However, in the mentioned studies,  
347 protein and carbohydrate-rich food were simultaneously available, which may have resulted  
348 in a negative contrast effect (Mitchell & Flaherty, 1998) such as the value of carbohydrate,  
349 relative to protein, was decreased as a function of the comparison, leading to increased  
350 protein consumption. Conversely, in the present study, rats have free access to a single option  
351 (protein-rich food), therefore the lack of comparison with carbohydrate might have resulted  
352 in no increased intake in PR rats. In line with this idea, the lack of increased intake of protein  
353 in PR rats in the absence of a choice between nutrients has been previously reported by our  
354 lab during conditioning and forced-choice sessions, when only one nutrient-rich solution was  
355 available (Chiacchierini et al., 2021; Murphy et al., 2018). Another possibility to explain the  
356 discrepancy between instrumental responding and free access results in Experiment 2 is that  
357 protein restriction had the effect of making rats less sensitive to the cost associated with the  
358 protein reinforcers, thereby elevating the threshold at which rats can sustainably exert effort.  
359 In behavioral economics, this effect is known as “inelastic” demand (Hursh & Silberberg,  
360 2008). It can finally be hypothesized that rats, over the 30-minute free access test, might have



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

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

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



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

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

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

- 416 Abey, N. O., Ebuehi, O. A. T., & Imaga, N. O. A. (2019). Neurodevelopment and Cognitive Impairment  
417 in Parents and Progeny of Perinatal Dietary Protein Deficiency Models. *Front Neurosci*, *13*,  
418 826. doi:10.3389/fnins.2019.00826
- 419 Almeida, S. S., Tonkiss, J., & Galler, J. R. (1996). Prenatal protein malnutrition affects exploratory  
420 behavior of female rats in the elevated plus-maze test. *Physiol Behav*, *60*(2), 675-680.  
421 doi:10.1016/s0031-9384(96)80047-3
- 422 Arnold, J. M., & Roberts, D. C. (1997). A critique of fixed and progressive ratio schedules used to  
423 examine the neural substrates of drug reinforcement. *Pharmacol Biochem Behav*, *57*(3), 441-  
424 447. doi:10.1016/s0091-3057(96)00445-5
- 425 Beeler, J. A., McCutcheon, J. E., Cao, Z. F., Murakami, M., Alexander, E., Roitman, M. F., & Zhuang, X.  
426 (2012). Taste uncoupled from nutrition fails to sustain the reinforcing properties of food. *Eur*  
427 *J Neurosci*, *36*(4), 2533-2546. doi:10.1111/j.1460-9568.2012.08167.x
- 428 Berridge, K. C. (2004). Motivation concepts in behavioral neuroscience. *Physiol Behav*, *81*(2), 179-209.  
429 doi:10.1016/j.physbeh.2004.02.004
- 430 Branch, S. Y., Goertz, R. B., Sharpe, A. L., Pierce, J., Roy, S., Ko, D., . . . Beckstead, M. J. (2013). Food  
431 restriction increases glutamate receptor-mediated burst firing of dopamine neurons. *J*  
432 *Neurosci*, *33*(34), 13861-13872. doi:10.1523/jneurosci.5099-12.2013
- 433 Chaumontet, C., Recio, I., Fromentin, G., Benoit, S., Piedcoq, J., Darcel, N., & Tomé, D. (2018). The  
434 Protein Status of Rats Affects the Rewarding Value of Meals Due to their Protein Content. *J*  
435 *Nutr*, *148*(6), 989-998. doi:10.1093/jn/nxy060
- 436 Chiacchierini, G., Naneix, F., Peters, K. Z., Apergis-Schoute, J., Snoeren, E. M. S., & McCutcheon, J. E.  
437 (2021). Protein appetite drives macronutrient-related differences in ventral tegmental area  
438 neural activity. *J Neurosci*. doi:10.1523/jneurosci.3082-20.2021
- 439 Clark, J. J., & Bernstein, I. L. (2006). Sensitization of salt appetite is associated with increased "wanting"  
440 but not "liking" of a salt reward in the sodium-deplete rat. *Behav Neurosci*, *120*(1), 206-210.  
441 doi:10.1037/0735-7044.120.1.206
- 442 Cone, J. J., McCutcheon, J. E., & Roitman, M. F. (2014). Ghrelin acts as an interface between  
443 physiological state and phasic dopamine signaling. *J Neurosci*, *34*(14), 4905-4913.  
444 doi:10.1523/jneurosci.4404-13.2014
- 445 Depoortere, R. Y., Li, D. H., Lane, J. D., & Emmett-Oglesby, M. W. (1993). Parameters of self-  
446 administration of cocaine in rats under a progressive-ratio schedule. *Pharmacol Biochem*  
447 *Behav*, *45*(3), 539-548. doi:10.1016/0091-3057(93)90503-I
- 448 Dickerson, F., Gennusa, J. V., 3rd, Stallings, C., Origoni, A., Katsafanas, E., Sweeney, K., . . . Yolken, R.  
449 (2020). Protein intake is associated with cognitive functioning in individuals with psychiatric  
450 disorders. *Psychiatry Res*, *284*, 112700. doi:10.1016/j.psychres.2019.112700
- 451 Du, F., Higginbotham, D. A., & White, B. D. (2000). Food intake, energy balance and serum leptin  
452 concentrations in rats fed low-protein diets. *J Nutr*, *130*(3), 514-521. doi:10.1093/jn/130.3.514
- 453 Eiselt, A.-K., Chen, S., Chen, J., Arnold, J., Kim, T., Pachitariu, M., & Sternson, S. M. (2021). Hunger or  
454 thirst state uncertainty is resolved by outcome evaluation in medial prefrontal cortex to guide  
455 decision-making.
- 456 Fotio, Y., Ciccocioppo, R., & Piomelli, D. (2021). N-acyl ethanolamine acid amidase (NAAA) inhibition  
457 decreases the motivation for alcohol in Marchigian Sardinian alcohol-preferring rats.  
458 *Psychopharmacology (Berl)*, *238*(1), 249-258. doi:10.1007/s00213-020-05678-7
- 459 Gould, J. M., Smith, P. J., Airey, C. J., Mort, E. J., Airey, L. E., Warricker, F. D. M., . . . Willaime-Morawek,  
460 S. (2018). Mouse maternal protein restriction during preimplantation alone permanently  
461 alters brain neuron proportion and adult short-term memory. *Proc Natl Acad Sci U S A*,  
462 *115*(31), E7398-e7407. doi:10.1073/pnas.1721876115
- 463 Grissom, N. M., & Reyes, T. M. (2013). Gestational overgrowth and undergrowth affect  
464 neurodevelopment: similarities and differences from behavior to epigenetics. *Int J Dev*  
465 *Neurosci*, *31*(6), 406-414. doi:10.1016/j.ijdevneu.2012.11.006

- 466 Hill, C. M., Laeger, T., Dehner, M., Albarado, D. C., Clarke, B., Wanders, D., . . . Morrison, C. D. (2019).  
467 FGF21 Signals Protein Status to the Brain and Adaptively Regulates Food Choice and  
468 Metabolism. *Cell Rep*, *27*(10), 2934-2947.e2933. doi:10.1016/j.celrep.2019.05.022
- 469 Hill, C. M., & Morrison, C. D. (2019). The Protein Leverage Hypothesis: A 2019 Update for Obesity.  
470 *Obesity (Silver Spring)*, *27*(8), 1221. doi:10.1002/oby.22568
- 471 Hodos, W. (1961). Progressive ratio as a measure of reward strength. *Science*, *134*(3483), 943-944.  
472 doi:10.1126/science.134.3483.943
- 473 Hursh, S. R., & Silberberg, A. (2008). Economic demand and essential value. *Psychol Rev*, *115*(1), 186-  
474 198. doi:10.1037/0033-295x.115.1.186
- 475 Jans, L. A., Korte-Bouws, G. A., Korte, S. M., & Blokland, A. (2010). The effects of acute tryptophan  
476 depletion on affective behaviour and cognition in Brown Norway and Sprague Dawley rats. *J*  
477 *Psychopharmacol*, *24*(4), 605-614. doi:10.1177/0269881108099424
- 478 Jewett, D. C., Cleary, J., Levine, A. S., Schaal, D. W., & Thompson, T. (1995). Effects of neuropeptide Y,  
479 insulin, 2-deoxyglucose, and food deprivation on food-motivated behavior.  
480 *Psychopharmacology (Berl)*, *120*(3), 267-271. doi:10.1007/bf02311173
- 481 Kriekhaus, E. E., & Wolf, G. (1968). Acquisition of sodium by rats: interaction of innate mechanisms  
482 and latent learning. *J Comp Physiol Psychol*, *65*(2), 197-201. doi:10.1037/h0025547
- 483 Laeger, T., Albarado, D. C., Burke, S. J., Trosclair, L., Hedgepeth, J. W., Berthoud, H. R., . . . Morrison, C.  
484 D. (2016). Metabolic Responses to Dietary Protein Restriction Require an Increase in FGF21  
485 that Is Delayed by the Absence of GCN2. *Cell Rep*, *16*(3), 707-716.  
486 doi:10.1016/j.celrep.2016.06.044
- 487 Laeger, T., Henagan, T. M., Albarado, D. C., Redman, L. M., Bray, G. A., Noland, R. C., . . . Morrison, C.  
488 D. (2014). FGF21 is an endocrine signal of protein restriction. *J Clin Invest*, *124*(9), 3913-3922.  
489 doi:10.1172/jci74915
- 490 Levitsky, D. A., Massaro, T. F., & Barnes, R. H. (1975). Maternal malnutrition and the neonatal  
491 environment. *Fed Proc*, *34*(7), 1583-1586.
- 492 Lutter, M., & Nestler, E. J. (2009). Homeostatic and hedonic signals interact in the regulation of food  
493 intake. *J Nutr*, *139*(3), 629-632. doi:10.3945/jn.108.097618
- 494 Markus, C. R. (2008). Dietary amino acids and brain serotonin function; implications for stress-related  
495 affective changes. *Neuromolecular Med*, *10*(4), 247-258. doi:10.1007/s12017-008-8039-9
- 496 Martel, P., & Fantino, M. (1996). Mesolimbic dopaminergic system activity as a function of food  
497 reward: a microdialysis study. *Pharmacol Biochem Behav*, *53*(1), 221-226. doi:10.1016/0091-  
498 3057(95)00187-5
- 499 McGaughy, J. A., Amaral, A. C., Rushmore, R. J., Mokler, D. J., Morgane, P. J., Rosene, D. L., & Galler, J.  
500 R. (2014). Prenatal malnutrition leads to deficits in attentional set shifting and decreases  
501 metabolic activity in prefrontal subregions that control executive function. *Dev Neurosci*,  
502 *36*(6), 532-541. doi:10.1159/000366057
- 503 Mitchell, C., & Flaherty, C. (1998). Temporal dynamics of corticosterone elevation in successive  
504 negative contrast. *Physiol Behav*, *64*(3), 287-292. doi:10.1016/s0031-9384(98)00072-9
- 505 Morrison, C. D., Reed, S. D., & Henagan, T. M. (2012). Homeostatic regulation of protein intake: in  
506 search of a mechanism. *Am J Physiol Regul Integr Comp Physiol*, *302*(8), R917-928.  
507 doi:10.1152/ajpregu.00609.2011
- 508 Morrison, C. D., Xi, X., White, C. L., Ye, J., & Martin, R. J. (2007). Amino acids inhibit *Agrp* gene  
509 expression via an mTOR-dependent mechanism. *Am J Physiol Endocrinol Metab*, *293*(1), E165-  
510 171. doi:10.1152/ajpendo.00675.2006
- 511 Murphy, M., Peters, K. Z., Denton, B. S., Lee, K. A., Chadchankar, H., & McCutcheon, J. E. (2018).  
512 Restriction of dietary protein leads to conditioned protein preference and elevated  
513 palatability of protein-containing food in rats. *Physiol Behav*, *184*, 235-241.  
514 doi:10.1016/j.physbeh.2017.12.011

- 515 Naneix, F., Peters, K. Z., & McCutcheon, J. E. (2020). Investigating the Effect of Physiological Need  
516 States on Palatability and Motivation Using Microstructural Analysis of Licking. *Neuroscience*,  
517 447, 155-166. doi:10.1016/j.neuroscience.2019.10.036
- 518 Olarte-Sánchez, C. M., Valencia-Torres, L., Cassaday, H. J., Bradshaw, C. M., & Szabadi, E. (2015).  
519 Quantitative analysis of performance on a progressive-ratio schedule: effects of reinforcer  
520 type, food deprivation and acute treatment with  $\Delta^9$ -tetrahydrocannabinol (THC). *Behav*  
521 *Processes*, 113, 122-131. doi:10.1016/j.beproc.2015.01.014
- 522 Pratt, W. E., Schall, M. A., & Choi, E. (2012). Selective serotonin receptor stimulation of the medial  
523 nucleus accumbens differentially affects appetitive motivation for food on a progressive ratio  
524 schedule of reinforcement. *Neurosci Lett*, 511(2), 84-88. doi:10.1016/j.neulet.2012.01.038
- 525 Quartermain, D., Miller, N. E., & Wolf, G. (1967). Role of experience in relationship between sodium  
526 deficiency and rate of bar pressing for salt. *J Comp Physiol Psychol*, 63(3), 417-420.  
527 doi:10.1037/h0024611
- 528 Raubenheimer, D., & Simpson, S. J. (2019). Protein Leverage: Theoretical Foundations and Ten Points  
529 of Clarification. *Obesity (Silver Spring)*, 27(8), 1225-1238. doi:10.1002/oby.22531
- 530 Richard, E. L., Laughlin, G. A., Kritz-Silverstein, D., Reas, E. T., Barrett-Connor, E., & McEvoy, L. K. (2018).  
531 Dietary Patterns and Cognitive Function among Older Community-Dwelling Adults. *Nutrients*,  
532 10(8). doi:10.3390/nu10081088
- 533 Roberts, D. C., Bennett, S. A., & Vickers, G. J. (1989). The estrous cycle affects cocaine self-  
534 administration on a progressive ratio schedule in rats. *Psychopharmacology (Berl)*, 98(3), 408-  
535 411. doi:10.1007/bf00451696
- 536 Robinson, M. J., & Berridge, K. C. (2013). Instant transformation of learned repulsion into motivational  
537 "wanting". *Curr Biol*, 23(4), 282-289. doi:10.1016/j.cub.2013.01.016
- 538 Rushmore, R. J., McGaughy, J. A., Amaral, A. C., Mokler, D. J., Morgane, P. J., Galler, J. R., & Rosene, D.  
539 L. (2021). The neural basis of attentional alterations in prenatally protein malnourished rats.  
540 *Cereb Cortex*, 31(1), 497-512. doi:10.1093/cercor/bhaa239
- 541 Sato, H., Tsukamoto-Yasui, M., Takado, Y., Kawasaki, N., Matsunaga, K., Ueno, S., . . . Kitamura, A.  
542 (2020). Protein Deficiency-Induced Behavioral Abnormalities and Neurotransmitter Loss in  
543 Aged Mice Are Ameliorated by Essential Amino Acids. *Front Nutr*, 7, 23.  
544 doi:10.3389/fnut.2020.00023
- 545 Schulkin, J. (1986). The evolution and expression of salt appetite. In *The physiology of thirst and sodium*  
546 *appetite* (pp. 491-496): Springer.
- 547 Sharma, S., Hryhorczuk, C., & Fulton, S. (2012). Progressive-ratio responding for palatable high-fat and  
548 high-sugar food in mice. *J Vis Exp*(63), e3754. doi:10.3791/3754
- 549 Simpson, S. J., & Raubenheimer, D. (2005). Obesity: the protein leverage hypothesis. *Obes Rev*, 6(2),  
550 133-142. doi:10.1111/j.1467-789X.2005.00178.x
- 551 Tobler, P. N., Fiorillo, C. D., & Schultz, W. (2005). Adaptive coding of reward value by dopamine  
552 neurons. *Science*, 307(5715), 1642-1645. doi:10.1126/science.1105370
- 553 Tonkiss, J., Foster, G. A., & Galler, J. R. (1991a). Prenatal protein malnutrition and hippocampal  
554 function: partial reinforcement extinction effect. *Brain Res Bull*, 27(6), 809-813.  
555 doi:10.1016/0361-9230(91)90213-4
- 556 Tonkiss, J., & Galler, J. R. (1990). Prenatal protein malnutrition and working memory performance in  
557 adult rats. *Behav Brain Res*, 40(2), 95-107. doi:10.1016/0166-4328(90)90002-v
- 558 Tonkiss, J., Galler, J. R., Shukitt-Hale, B., & Rocco, F. (1991b). Prenatal protein malnutrition impairs  
559 visual discrimination learning in adult rats. *J Psychobiology*, 19(3), 247-250.
- 560 Vargas, M. A., Luo, N., Yamaguchi, A., & Kapahi, P. (2010). A role for S6 kinase and serotonin in  
561 postmating dietary switch and balance of nutrients in *D. melanogaster*. *Curr Biol*, 20(11), 1006-  
562 1011. doi:10.1016/j.cub.2010.04.009
- 563 Velázquez-Sánchez, C., Ferragud, A., Moore, C. F., Everitt, B. J., Sabino, V., & Cottone, P. (2014). High  
564 trait impulsivity predicts food addiction-like behavior in the rat. *Neuropsychopharmacology*,  
565 39(10), 2463-2472. doi:10.1038/npp.2014.98

- 566 Vendruscolo, L. F., Gueye, A. B., Darnaudéry, M., Ahmed, S. H., & Cador, M. (2010). Sugar  
567 overconsumption during adolescence selectively alters motivation and reward function in  
568 adult rats. *PLoS One*, 5(2), e9296. doi:10.1371/journal.pone.0009296
- 569 White, B. D., Porter, M. H., & Martin, R. J. (2000). Effects of age on the feeding response to moderately  
570 low dietary protein in rats. *Physiol Behav*, 68(5), 673-681. doi:10.1016/s0031-9384(99)00229-  
571 2
- 572 Wu, Y., Li, B., Li, L., Mitchell, S. E., Green, C. L., D'Agostino, G., . . . Speakman, J. R. (2021). Very-low-  
573 protein diets lead to reduced food intake and weight loss, linked to inhibition of hypothalamic  
574 mTOR signaling, in mice. *Cell Metab*, 33(6), 1264-1266. doi:10.1016/j.cmet.2021.04.016
- 575 Zapata, R. C., Singh, A., Pezeshki, A., Avirineni, B. S., Patra, S., & Chelikani, P. K. (2019). Low-Protein  
576 Diets with Fixed Carbohydrate Content Promote Hyperphagia and Sympathetically Mediated  
577 Increase in Energy Expenditure. *Mol Nutr Food Res*, 63(21), e1900088.  
578 doi:10.1002/mnfr.201900088
- 579