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1 Dietary proteins improve hibernation and subsequent reproduction
2 in the European hamster, *Cricetus cricetus*
3

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7

8 Abbreviations: Body temperature: Bt, Fat-Free Mass: FFM, Fat Mass: FM, Linear Mixed
9 Model: LMM, Polyunsaturated fatty acids: PUFA, Total Body Water: TBW, Unsaturated
10 fatty acids: UFA, Saturated Fatty Acids: SFA, Water/protein ratio: W/P.

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12 Running head: Proteins improve hibernators' lifecycle

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24 **Abstract**

25 The reproductive success of hibernators depends not only on food quality during
26 reproduction, but also on their body condition when emerging after hibernation, which in turn
27 is highly dependent on the hibernation pattern. To date, no studies have fully investigated the
28 role of macronutrients throughout the annual lifecycle of hibernators and the strong
29 interdependency between its different phases. This study tested the effects of two diets with
30 different lipid and protein composition on the pre-hibernation body condition, hibernation
31 pattern and reproduction of captive-reared European hamsters. Hamsters fed the high-lipid
32 diet gained more body mass in the pre-hibernation period than those eating the high-protein
33 diet, spent less time in torpor and thus lost more body mass during hibernation. Despite
34 similar body conditions in both groups at the start of reproduction, the group fed the high-
35 protein diet had higher reproductive success, with more numerous and larger pups than in the
36 high-lipid group. This study reveals that the macronutrient content of diets affects the
37 different phases of the annual lifecycle in hamsters, each of which affects the next. Thus, a
38 high-lipid diet induces less torpor use during hibernation due to a better pre-hibernation body
39 condition, and negatively impacts subsequent reproduction.

40

41 **Background**

42 Some mammals face the seasonal limitations in food and water availability by using specific
43 adaptive strategies such as hibernation (20). Hibernation consists of a succession of multiday
44 torpor bouts, interspersed with periods of euthermia (33). During torpor, hibernating
45 mammals reduce their metabolism and body temperature, and many physiological functions
46 cease (20, 33). To prepare for the hibernation season and thus overcome the harsh winter
47 conditions, these animals have to store large energy reserves during late summer and fall.
48 Food-storing species hoard food in a burrow, which is consumed during hibernation, while
49 fat-storing species build up high body fat reserves for their needs during the winter (22).
50 For a hibernator, each phase of the annual cycle is strongly dependent on the previous one.
51 Therefore, poor stockpiling *prior* to hibernating may induce an extended use of torpor to
52 ensure energy sparing, resulting in delayed emergence from hibernation and impaired body
53 condition (27). Animals with poor body conditions on emergence generally display low
54 reproductive success, i.e. reduced mating opportunities for males and fewer litters for females
55 (8). Moreover, their late emergence from hibernation gives them less time to hoard food for
56 the next hibernation period, thus jeopardizing the entire annual cycle to come. The quality of
57 the reserves stored during the pre-hibernation period is therefore a key factor determining the
58 survival and reproduction of hibernators (11, 16, 17). Dietary lipids are crucial to the rapid
59 constitution of high-energy reserves in fat-storing species, and also enable food-storing
60 hibernators to maintain their body condition and overwinter survival (8). Previous literature
61 states that the degree of unsaturation of dietary fatty acids determines hibernation quality in
62 fat-storing species. Indeed, polyunsaturated fatty acids (PUFA) lead to a reduction in the
63 minimum body temperature set-point and the minimum metabolic rate through the
64 improvement of torpor bout duration and depth (11, 26). However, PUFA are more prone to
65 peroxidation than saturated lipids, and an excess thereof in the diet of a hibernator can reverse

66 their positive effects on hibernation quality (13, 15). Based on these results, it seems that an
67 optimum rather than a maximum quantity of PUFA is beneficial for hibernation (13, 14). The
68 type of PUFA is also crucial; an n-3 linolenic acid-enriched diet prevents hibernation
69 behaviour (21), whereas an n-6 linoleic acid-enriched diet improves torpor bout length and
70 depth (18, 26). Food-storing hibernators seem to be less affected by the degree of unsaturation
71 of fatty acids in the diet, as observed in the Turkish hamster (*Mesocricetus brandti*) (2). The
72 shorter torpor bouts occurring in food-storing species may lead them to be less affected by
73 lipid peroxidation than fat-storing hibernators (2).

74 Macronutrient food composition has also been widely demonstrated to impact reproduction
75 and juvenile growth rate in a large range of species including hibernators. Their high energy
76 supply efficiency demands sufficient lipids to cover the high energy demands of mating,
77 gestation and lactation (35), especially in small mammals living in highly seasonal
78 environments (23). In hibernators, triglycerides have been shown to accelerate sexual
79 maturation at emergence. This has been shown in juvenile golden-mantled ground squirrels
80 (*Spermophilus lateralis*), in which a high-fat diet induces higher testes masses and plasma
81 testosterone concentrations after hibernation (7). Moreover, PUFA also specifically increase
82 testosterone concentrations in the European ground squirrel (*Spermophilus citellus*), (36). Diet
83 composition is also important during lactation (23, 38). This has been illustrated in chipmunks
84 (*Tamias sp.*), where the milk energy value doubles between the beginning and the end of
85 lactation as it is progressively enriched in lipids and proteins until they constitute 30 % and 10
86 % of its wet mass, respectively (38).

87 The present study aimed to evaluate the effects of two diets with different lipid and protein
88 contents (see Tab.1 for macronutrient composition) on the annual lifecycle of a food-storing
89 hibernator, the European hamster (*Cricetus cricetus*). In a first step, we investigated the
90 effects of these two diets on hamster feeding behaviour and body condition during the pre-

91 hibernation period (from June to September), on their hibernation pattern and on their body
92 condition at emergence in January. We predicted that animals fed a high-lipid / low-protein
93 diet would (1) show a higher body mass and fat mass gain before hibernation due to a higher
94 energy intake than the high-protein / low-lipid group. As a consequence, we hypothesized that
95 they would (2) spend less time in torpor and (3) lose more body mass during hibernation, but
96 would (4) still show a better body condition at emergence than the second diet group.
97 Secondly, we compared the effects of the two diets on the reproductive success of European
98 hamsters. We predicted that (5) the adult animals with the best body condition at emergence
99 from hibernation (i.e. the individuals fed a high-lipid / low-protein diet, according to our 4th
100 hypothesis) would have the best reproductive success (more pups per female with a higher
101 body mass at birth), but that (6) juveniles from the high-protein diet would catch up for
102 growth delay in the first weeks after birth.

103

104 **Methods**

105 *Animals & diets*

106 A total of 94 one-year-old (at the beginning of the experiment) European hamsters were used
107 for the experiments. These were separated as follows: 34 females for the experiment on body
108 composition (see Section A: ‘Diet and hibernation’), and 30 breeding pairs for the
109 reproduction experiment (see Section D: Diet and reproduction quality’). Animals were
110 maintained under photoperiodic and thermal conditions that mimicked an annual cycle (39).
111 Hibernation in this species is controlled by an endogenous circannual clock (25) and occurred
112 under controlled photoperiod in captivity from mid-October to the end of January. They were
113 housed individually for the entire period except for a two-week reproduction period, in which
114 they were housed as breeding pairs.

115 Animals had free access to water and food in both experiments. One group of 47 individuals
116 was fed with a protein-enriched diet (high-protein group) and the remaining 47 hamsters were
117 fed a lipid-enriched diet (high-lipid group) (Safe, Augy, France; see Tab.1 for macronutrient
118 composition). The high-lipid diet contained twice as much lipids as the high-protein diet. The
119 protein content was adjusted in order to have identical energy and carbohydrate content in
120 both diets. Pellets had the same shape, size and hardness in both diets. Animals were fed these
121 diets from June 2013 (beginning of the experiment, starting with a pre-hibernation stockpiling
122 phase and then throughout hibernation and reproduction) to August 2014 (end of monitoring
123 of pup growth).

124 The experimental protocol followed the EU Directive 2010/63/EU for animal experiments and
125 the care and use of laboratory animals, and was approved by the Ethical Committee under
126 agreement number 00624.01.

127 *Experimental procedures*

128 *A. Diet and hibernation*

129 Thirty-four females were used for this first study (mean body mass = 286.9 ± 6.3 g) and were
130 randomly assigned to one of the two nutritional groups, i.e. the high-protein or the high-lipid
131 diet. Food consumption was determined weekly by weighing the pellets (± 0.1 g) and
132 calculating the difference between the amount of pellets they received and the amount
133 remaining. Animals were weighed monthly (± 0.1 g), except during hibernation to avoid any
134 disturbance of the animals. Fourteen females (7 per diet) were euthanized *prior* to hibernation
135 in September 2013 (pre-hibernation groups) and the 20 remaining females (10 per diet) were
136 euthanized in January 2014, *i.e.* at the end of hibernation and before emergence (post-
137 hibernation groups).

138 *B. Hibernation pattern assessment*

139 In June 2013, a temperature logger (iButton®, DS1922L model, Maxim Integrated Products,
140 Sunnyvale, CA, USA, weighing 3.2 ± 0.1 g after paraffin ELVAX® inclusion) was implanted
141 intraperitoneally in each of the 20 females assigned to the post-hibernation groups to monitor
142 changes in body temperature throughout hibernation. Surgical procedures were performed
143 according to Paul et al. (29) excepting that isoflurane anesthesia was used (2.5% in $1.2 \text{ L} \cdot \text{min}^{-1}$
144 1 oxygen). Animals were considered to be hypothermic when their body temperature dropped
145 below 32°C (29).

146 *C. Body composition assessment*

147 After euthanasia by decapitation under 2% isoflurane anaesthesia, the carcass was weighed
148 (± 0.01 g) and the digestive tract content was removed. The carcass was weighed again and cut
149 into small pieces before being freeze-dried to constant mass and ground under liquid nitrogen
150 to obtain a homogenous powder for analysis. Prior to analyses, the powder was lyophilized
151 for 48h to eliminate any remaining traces of water. Nitrogen content was determined in

152 triplicate according to the Kjeldahl method using 150-200mg aliquots. Protein content was
153 calculated as nitrogen x 6.25 (5). Lipid content was determined in duplicate using 1g aliquots
154 according to a procedure adapted from the Folch method (12) and using a
155 chloroform/methanol solution (2/1, v/v) as extraction solvents. Ash content was determined
156 gravimetrically in duplicate from 1-2g samples ignited in a muffle furnace at 400°C for 24h.
157 Total Body Water (TBW) was calculated by subtracting total dry body mass from fresh body
158 mass. Fat-Free Mass (FFM) was the sum of TBW, protein content and ash content.

159 *D. Diet and reproduction quality*

160 Thirty mating pairs (15 per diet) were used for this second experiment (average body mass of
161 224.9±8.7 and 195.2±8 respectively for males and females prior to emergence from
162 hibernation in January). When assigning animals to each diet group, care was taken not to
163 assign animals to groups containing their kin. Mean body mass was similar between groups at
164 the start of the experiment. Animals were housed and hibernated in the same conditions as in
165 the first study (see Section A: ‘Diet and hibernation’). Six weeks after the end of hibernation
166 (*i.e.* in March 2014), males and females in each group were put into mating pairs for 2 weeks.
167 A plastic shelter was placed in each mating cage to limit intra-pair conflicts. At the end of the
168 mating period, males were removed and the cages of the females were checked every day to
169 determine the litter birth date. Gestation lasts 15-18 days in captivity (10). If no birth occurred
170 two weeks after the separation from males, females were weighed to determine if they were
171 pregnant. If they were not pregnant, they were mated with another male. Females that had
172 successfully mated weaned their pups at the age of 28 days, and were mated for a second time
173 with a different male after three days of rest. The number of successful matings was recorded.
174 From seven days post-partum, pups were weighed (±0.1 g) every three days until weaning,
175 when they were separated from their mothers. Pups were then fed the same diet as their
176 mothers and were weighed every seven days, up to 105 days *post-partum*.

177

178 *Statistical analyses*

179 The data presented are mean values \pm SD or SEM. For the first experiment (effects of the diet
180 on the body mass, body composition, food intake and patterns of hibernation), a Mann-
181 Whitney U-test was performed to test the significance of the difference ($p < 0.05$) in mean
182 body mass, fat-mass, fat-free mass and fat-free mass components (TBW, protein content and
183 ash content) between the diets. Linear Mixed Models (LMMs) were used to investigate the
184 effects of the diet on daily food, energy, protein, lipid, PUFA and SFA intakes and on body
185 mass change from June to January in adult females. Diet, month and diet*month interactions
186 were included as fixed factors. The identity of the individuals was included as a random factor
187 for repeated measurements on the same individuals. When examining the effect of the diet on
188 the food intake of hamsters, their body mass at the beginning of the month was included as a
189 covariate. The effects of each diet on the hibernation pattern, namely (i) the date of the first
190 torpor event, (ii) the number of torpor bouts, (iii) the total time spent in torpor, (iv) the mean
191 duration of torpor bouts, (v) the minimal body temperature, (vi) the number of euthermic
192 phases, (vi) the mean duration of euthermic phases and (vii) the total time during which the
193 animal was euthermic were analysed using Mann-Whitney U-tests.

194 The effect of the diet on reproductive success was investigated using linear models (after
195 verifying for the normality of the residuals). The effects of each diet on the total number of
196 litters per female (i_2) at parturition and (ii_2) at weaning was analysed using a linear model,
197 with the diet group as a fixed factor. An LMM was used to investigate how each diet affected
198 litter size (iii_2) at parturition and (iv_2) at weaning, with the diet group, the reproduction
199 number and the diet*reproduction interaction used as fixed factors. The identity of the
200 females was included as a random factor to control for repeated measures on the same
201 individuals. Finally, the effects of each diet on pup body mass growth was also investigated

202 using an LMM. The diet type, the litter number (first or second), the age and the sex of the
203 pups were included as fixed factors, as well as the four following interactions: sex*age,
204 sex*diet, age*diet and sex*age*diet. The litter size and the mother's body mass were both
205 included as covariates. The identity of the pups, nested in the identity of the litter, was
206 included as a random factor for repeated measurements on the same individuals and the same
207 litters. Only pups that survived until weaning were included in this analysis. For all the LMM,
208 the normality of the residuals was tested using a Kolmogorov-Smirnov test and variance
209 homogeneity was checked using a Levene test. Multiple comparisons were analyzed via post-
210 hoc LSD (least significant difference) testing. The final model selection was based on the best
211 AICc (Akaike information criterion for small samples) value. Analyses were conducted using
212 IBM SPSS software (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM
213 Corp), and the significance threshold was set at $p < 0.05$. Figures were prepared using
214 GraphPad prism software (Version 5, La Jolla, USA).

215

216 **Results**

217 *The influence of each diet on the body condition of adult females*

218 The effect of the diets on the body mass of adult females varied significantly according to the
219 month ($F_{4,430.4}=240.7$, $p<0.001$, increasing from June to July, then decreasing during
220 hibernation, *i.e.* -80.9 ± 4.1 g from September to January, $p<0.01$, Fig. 1A). However, no
221 significant effect of the diet alone was found on the overall body mass of the females during
222 this experiment ($F_{1,31.8}=3.3$, $p=0.08$). A significant diet*month interaction was, however,
223 observed ($F_{4,430.4}=25.2$, $p<0.001$). Post-hoc analyses highlighted that there was no effect of the
224 diet on the body mass of females at the beginning (June, high-protein group = 287.7 ± 5.1 g,
225 high-lipid group = 290.3 ± 5.3 g, $p = 0.73$) and at the end of this first experiment (January,
226 high-protein group = 187.7 ± 9.5 g, high-lipid group = 196.5 ± 9.5 g, $p = 0.53$). However, from
227 July to September (pre-hibernation period), the hamsters in the high-lipid group had a
228 significantly higher body mass than the hamsters that were fed the high-protein diet (by
229 22.6 ± 6.1 g in July, 34.8 ± 6.5 g in August and 42.2 ± 11.6 g in September, $p\leq 0.001$). The high-
230 lipid group gained more fat-free mass (Fig. 1C) (Mann-Whitney U-test $p=0.002$) and
231 particularly body proteins and water (Mann-Whitney U-test: $p=0.007$, $p=0.002$, respectively),
232 but the water/protein ratio (W/P) was similar in the two groups (high-protein group =
233 2.76 ± 0.02 and high-lipid group = 2.84 ± 0.04 , Mann-Whitney U-test $p=0.11$). Fat mass was not
234 affected by diets (Fig. 1B) (Mann-Whitney U-test $p= 0.46$). The ash component was not
235 significantly different between groups in September (Mann-Whitney U-test $p=0.06$) (Fig. 1C).
236 During hibernation, animals from the high-lipid group displayed a greater body mass loss
237 ($35\pm 1\%$ of the pre-hibernation mass, Fig. 1A) than that observed in the high-protein group
238 ($27\pm 1\%$ of the pre-hibernation mass) (Mann-Whitney U-test $p=0.004$). Lipids contributed the
239 most to the mass loss during hibernation with a 66% decrease in both groups (Fig. 1B). The
240 high-lipid group also presented a decrease in ash and protein masses during this period (-12%

241 for ash, $p=0.037$ and -26% for protein, $p=0.001$ (Wilcoxon test), Fig. 1C). Consequently, the
242 W/P value decreased significantly in the high-lipid group during hibernation, from 2.84 ± 0.04
243 to 2.66 ± 0.04 (Wilcoxon test: $p=0.01$), whereas no change was observed in the high-protein
244 group (ranging between 2.76 ± 0.02 and 2.68 ± 0.04). At the end of hibernation, protein, ash and
245 water contents as well as W/P values were similar in both groups (Mann-Whitney U-test,
246 respectively $p=0.15$, $p=0.96$, $p=0.15$, $p=0.82$).

247

248 *Influence of the diets on the food consumption of adult females*

249 Daily food intake was significantly affected by the type of diet ($F_{1,32.4} = 5.3$, $p = 0.03$), the
250 month ($F_{3,88.5} = 15.9$, $p < 0.001$), the diet*month interaction ($F_{3,86.8} = 4.1$, $p = 0.009$) and the
251 body mass of the individuals ($F_{1,54.4} = 11.5$, $p = 0.001$). On average, individuals in the high-
252 protein group ingested less food (7.4 ± 1.0 g.day⁻¹) than individuals in the high-lipid group
253 (8.3 ± 1.0 g.day⁻¹; mean difference = -0.92 ± 0.40 g.day⁻¹). All females in both groups ingested
254 on average more food in June and July (8.7 ± 1.0 g.day⁻¹ in June and 8.3 ± 1.0 g.day⁻¹ in July, no
255 significant differences: $p = 0.12$) than in August and September (7.5 ± 1.0 g.day⁻¹ in August
256 and 6.8 ± 1.0 g.day⁻¹ in September, $p < 0.05$, Fig. 2A). Post-hoc tests of the diet*month
257 interaction highlighted that the only significant difference between the high-protein and high-
258 lipid groups occurred in June (Fig. 2.A., $p = 0.046$, $N = 17$ per group; high-protein group =
259 8.2 ± 1.0 g.day⁻¹ and high-lipid group = 9.2 ± 1.0 g.day⁻¹), but not from July to September ($N =$
260 17 per group; $p \geq 0.4$). Overall, during the pre-hibernation phase, food intake decreased by
261 $\sim 30\%$ in the high-protein group (reaching 5.9 ± 1.1 g.day⁻¹ in September), whereas it decreased
262 by only $\sim 15\%$ in the high-lipid group (reaching 7.8 ± 1.1 g.day⁻¹ in September). Due to similar
263 energetic density of diets, the daily energy intake followed a similar trend to that of the daily
264 food intake (Fig. 2B) and was also significantly affected by the diet type ($F_{1,32.3} = 13.5$, $p =$
265 0.001), the month ($F_{3,80.7} = 15.2$, $p < 0.001$) and the diet*month interaction ($F_{3,80.7} = 6.3$, $p =$

266 0.001). Females from the high-protein group ingested on average significantly less energy per
267 day ($122.6 \pm 20.8 \text{ kJ} \cdot \text{day}^{-1}$) than females from the high-lipid group ($151.1 \pm 20.9 \text{ kJ} \cdot \text{day}^{-1}$). All
268 females in both groups ingested significantly less energy per day from June to September
269 (from $149.7 \pm 20.6 \text{ kJ} \cdot \text{day}^{-1}$ in June to $116.3 \pm 20.8 \text{ kJ} \cdot \text{day}^{-1}$ in September, $p < 0.001$). Post-hoc
270 analyses of the diet*month interaction (Fig. 2B) highlighted that females from the high-
271 protein group ingested less energy than females from the high-lipid group in June (Mean
272 difference = $-22.3 \pm 9.3 \text{ kJ} \cdot \text{day}^{-1}$, $p = 0.023$), August (Mean difference = $-31.6 \pm 10.5 \text{ kJ} \cdot \text{day}^{-1}$, p
273 = 0.005) and September (Mean difference = $-51.2 \pm 15.1 \text{ kJ} \cdot \text{day}^{-1}$, $p = 0.003$) but not in July
274 (Mean difference = $-8.2 \pm 6.8 \text{ kJ} \cdot \text{day}^{-1}$, $p = 0.2$).

275 Daily protein intake was also significantly influenced by the diet type ($F_{1,32.1} = 19.2$, $p < 0.001$),
276 the month ($F_{3,80.5} = 19.0$, $p < 0.001$) and the diet*month interaction ($F_{3,80.5} = 10.7$, $p < 0.001$).
277 Individuals from the high-protein group ingested on average more proteins than individuals
278 from the high-lipid group (Fig. 2C; mean difference of $-0.32 \pm 0.07 \text{ g} \cdot \text{day}^{-1}$). Post-hoc analyses
279 on the diet*month interaction revealed significant inter-group differences in June ($p < 0.001$),
280 July ($p < 0.001$) and August ($p = 0.009$) but not in September ($p = 0.99$). Protein ingestion was
281 reduced in September in the high-protein group, while it remained constant in the high-lipid
282 group (Fig. 2C).

283 For total daily lipid intake, we found an effect of diet type ($F_{1,32.5} = 170.9$, $p < 0.001$), and
284 month ($F_{3,80.8} = 9.5$, $p < 0.001$) but no effect was observed for the diet*month interaction
285 ($F_{3,80.8} = 2.3$, $p = 0.08$). Females from the high-protein group ingested on average half as much
286 lipids as females from the high-lipid group on a daily basis (Fig. 2D; mean difference = $-$
287 $0.475 \pm 0.036 \text{ g} \cdot \text{day}^{-1}$), whatever the month. Regarding the effects of the month, daily lipid
288 intake significantly decreased in both groups from June to September (from $0.66 \pm 0.10 \text{ g} \cdot \text{day}^{-1}$
289 in June to $0.53 \pm 0.1 \text{ g} \cdot \text{day}^{-1}$ in September, $p < 0.001$). Separate consideration of the saturated
290 fatty acid (SFA) and the polyunsaturated fatty acid (PUFA) intakes showed that SFA intake

291 was significantly affected by the diet type ($F_{1,32.6} = 294.6$, $p < 0.001$) and the month ($F_{3,80.9} =$
292 6.8 , $p < 0.001$) but not by the diet*month interaction ($F_{3,80.9} = 1.4$, $p = 0.26$), with females from
293 the high-protein group ingesting less SFA on average than females from the high-lipid group
294 (Fig. 2E; mean difference = -0.184 ± 0.011 g.day⁻¹), and with a significant decrease in SFA
295 daily intake from June to September (mean difference = -0.031 ± 0.007 g.day⁻¹, $p < 0.001$). The
296 daily PUFA intake was affected by the diet type ($F_{1,32.5} = 127.8$, $p < 0.001$), the month ($F_{3,80.8} =$
297 10.6 , $p < 0.001$) and the diet*month interaction ($F_{3,80.8} = 2.9$, $p = 0.041$), with individuals from
298 the high-protein group ingesting on average less PUFA than individuals from the high-lipid
299 group (Fig. 2F; mean difference = -0.291 ± 0.026 g.day⁻¹) and showed a decrease in the PUFA
300 intake from June to September (mean difference = -0.093 ± 0.017 g.day⁻¹, $p < 0.001$). Post-hoc
301 analyses of the diet*month interaction (Fig. 2F) highlighted that females from the high-
302 protein group ingested less PUFA than females from the high-lipid group whatever the month,
303 although the difference between the two groups was highest in September [i.e. mean
304 difference = -0.33 ± 0.05 g.day⁻¹, $p < 0.001$, compared to August (mean difference = -0.30 ± 0.03
305 g.day⁻¹, $p < 0.001$), June (mean difference = -0.29 ± 0.03 g.day⁻¹, $p < 0.001$) and July (mean
306 difference = -0.24 ± 0.02 g.day⁻¹, $p < 0.001$)].

307

308 *Diet influence on pre-hibernation and hibernation behaviour*

309 Some animals in the high-protein group displayed daily torpor during the pre-hibernation
310 period in July and August, a behaviour that was not observed in the high-lipid group. During
311 hibernation (from September onwards), the high-protein group displayed significantly more
312 torpor bouts than the high-lipid group (Mann-Whitney U-test $p = 0.013$, Tab. 2), but the mean
313 duration of torpor bouts was similar in both groups ($p = 0.21$, Tab. 2). Finally, the total time
314 spent in torpor was significantly higher in the high-protein than in the high-lipid group
315 ($p = 0.015$, Tab. 2). No differences appeared in the minimal body temperature reached during

316 torpor (high-protein group = $7.34 \pm 0.04^\circ\text{C}$ and high-lipid group = 7.38 ± 0.04 , Mann-Whitney
317 U-test $p=0.53$). Finally, the high-lipid group had significantly fewer and shorter euthermic
318 phases than the high-protein group ($p = 0.004$, Tab. 2). As a result, the total duration of
319 euthermia for animals in the high-lipid group was significantly shorter than that observed in
320 the high-protein group ($p = 0.001$, Tab. 2). In total, the high-protein group hibernated longer
321 than the high-lipid group (3144.3 ± 77.5 hours for high-protein group and 2548.2 ± 99.2 hours
322 for high-lipid group, Mann-Whitney U-test $p = 0.006$). In addition, the time spent in torpor
323 ($Bt < 32^\circ\text{C}$) during hibernation in high-protein and high-lipid groups was inversely correlated
324 with the body mass prior to hibernation (for all animals, $Rho=-0.845$, $p<0.001$).

325

326 *Diet influence on reproduction*

327 All variables that were representative of the reproductive success were significantly affected
328 by the diet, except for the average number of litters at parturition ($F_{1;22} = 1.4$, $p = 0.25$; Tab.
329 3). The diet had a significant effect on the number of litters at weaning ($F_{1;22} = 5.9$, $p = 0.024$;
330 Tab. 3). Results of the LMM regarding the litter size at parturition revealed a significant effect
331 of the diet ($F_{1;23} = 15.1$, $p = 0.001$; Tab. 3), but showed no effect of the litter number ($F_{1;22} =$
332 0.1 , $p = 0.8$; first litter: 4.9 ± 0.9 and second litter: 5.1 ± 1.0 pups per female) or the diet*litter
333 number interaction ($F_{1;22} = 0.2$, $p = 0.6$). The best model for the litter size at weaning was that
334 with only the diet group as a fixed factor, which was found to significantly affect the response
335 variable ($F_{1;22} = 28.7$, $p < 0.001$; Tab. 3). The significant effect of the diet group on this
336 variable was also confirmed in the other models that were less robust.

337

338 *The influence of diet on the body mass of pups*

339 The body mass of pups was significantly affected by all variables included in the LMM
340 (namely age, diet, sex, litter size, body mass of mothers, and interactions age*sex*diet: $5.5 < F$

341 <2400, $P < 0.001$) except the litter number ($F = 0.1$, $P = 0.77$). On average, males were heavier
342 than females, but this effect was dependent upon the age and the diet. The effects of this
343 Sex*Age*Diet group interaction on the body mass of pups are shown in Fig. 3. Post-hoc
344 analyses highlighted that males in the high-protein group were significantly heavier than
345 males from the high-lipid group throughout the experiment (i.e. from the age of seven to 105
346 days, see Fig. 3). However, males in the high-protein group started to differ from females in
347 the same group at the age of just 28 days (Fig. 3). Females in the high-protein group were
348 heavier than females in the high-lipid group throughout the weighing period, i.e. from seven
349 to 105 days of age. However, they only differed from males of the high-lipid group between
350 the ages of 13 and 63 days-old (Fig. 3). Finally, males from the high-lipid group were heavier
351 than females from the same group from the age of 42 to 105 days (see Fig. 3).

352 **Discussion**

353 This study is the first to investigate the role of proteins versus lipids on the hibernation
354 behaviour and reproductive success of a food-storing hibernating species. Our main results
355 show that, compared to a high-protein/low-lipid diet, a high-lipid/low-protein diet induced
356 changes in the management of energy reserves during pre-hibernation and hibernation
357 periods: hamsters fed a high-lipid diet had a higher body mass before hibernation than the
358 high-protein group, spent then less time in torpor and thus lost more body mass during
359 hibernation (Fig. 4). This resulted in a reduced reproductive success of adult females and
360 slower growth rate of pups in the high-lipid group.

361 The literature describes many factors influencing the duration and depth of torpor bouts
362 during hibernation. One of these factors is an optimal PUFA content of the diet before
363 hibernation, which decreases the minimum body temperature set-point, prolongs torpor bout
364 duration and attenuates hibernation mass loss (26, 32). Animals from the high-protein and
365 high-lipid groups remained in the optimal consumption range for PUFA throughout the
366 experiment; this hypothesis cannot therefore be considered to explain the differences we
367 observed in torpor bout duration. However, the greater ingestion of lipids and SFA by the
368 high-lipid group in summer and before hibernation could explain the reduced use of torpor, as
369 observed in the Turkish hamster (*Mesocricetus brandti*) (2).

370 Nevertheless, in accordance with previous studies in fat-storing species (3, 41) and in line
371 with our prediction, the time European hamsters spent in torpor ($Bt < 32^{\circ}\text{C}$) during hibernation
372 was inversely correlated with body mass prior to hibernation. Similarly, in food-storing
373 species under natural conditions, animals with the largest food hoards spent less time in torpor
374 (22, 27). These data suggest that since torpor is experienced to face food scarcity, huge energy
375 reserves, whether they are internal and/or external, might render the torpor strategy less
376 useful.

377 In line with our prediction, animals that were fed the high-lipid diet were heavier prior to
378 hibernation than the animals that ate the high-protein diet. Surprisingly, this difference in
379 body mass was mainly due to higher levels of protein and total body water compartments, and
380 not fat mass. An explanation could be that the high-lipid group had a better protein
381 assimilation rate due to a higher lipid intake that would have slowed down the intestinal
382 transit. Alternatively, these results could be explained by differences in fuel partitioning
383 related to differences in activity patterns: daily torpor bouts were already observed in the
384 high-protein group during the pre-hibernation period, whereas animals fed the high-lipid diet
385 stayed active all the time and could thus maintain a higher muscular development before
386 hibernation. Despite a lower lipid intake before hibernation, animals fed the high-protein diet
387 had a similar fat mass before hibernation to that of animals fed the high-lipid diet. This could
388 also be explained by the fact that the daily torpor of animals from the high-protein group
389 before hibernation could have allowed them to maximize lipid storage. Such behaviour has
390 previously been observed in a bat species and was seen to facilitate the constitution of
391 sufficient body reserves for hibernation (34). Furthermore, animals of the high-protein group
392 may have reduced their protein ingestion before hibernation to minimize possible negative
393 effects of protein on hibernation, as hypothesized by Humphries et al. (22).

394 In line with our prediction, animals of the high-lipid group spent less time in torpor during
395 hibernation and lost significantly more body mass than those from the high-protein group.
396 They mainly lost fat reserves, to a similar extent to that of hamsters in the high-protein group,
397 but their body protein and mineral masses also significantly decreased during hibernation, as
398 expected given the lack of proteins in their diet. However, contrary to our prediction, the two
399 groups had the same body mass and body composition at emergence from hibernation, despite
400 different hibernating behaviours. Animals exposed to the diet with the higher protein ratio
401 displayed a greater number of torpor bouts and more inter-bout euthermia. Interestingly, even

402 if their hibernation behaviour tended to maximize energy sparing, their inter-bout euthermia
403 lasted longer, probably to ensure functions that require euthermia after long torpor bouts such
404 as metabolic waste excretion, especially of urea (24, 28). To sum up, although food quality
405 modifies food intake and body composition, animals manage their torpor use and manage
406 their body reserves in such a way that at emergence (*i.e.* at the beginning of the reproductive
407 season) animals in both groups have the same body composition whatever the diet.

408 Despite similar body composition, the two groups did not have the same reproductive output,
409 indicating that the macronutrient composition of the diet directly impacted reproduction. Yet
410 contrary to our prediction, the high-lipid group had the lowest reproductive success. We did
411 not observe any significant difference in the average date of first parturition, but the number
412 of successful matings was lower in the high-lipid group. This result could be explained by
413 delayed sexual maturity for some of the individuals from the high-lipid group, as previously
414 observed in protein-restricted animals (4, 19). A lower litter size was also recorded in the
415 high-lipid group. However, since litter size was not measured before the pups were 7 days old
416 to avoid any disturbance and stress for the mothers, the real number of pups at parturition was
417 unknown. The difference observed in the size of litters between the high-protein and high-
418 lipid groups could either be explained by a production of smaller litters by females from the
419 high-lipid group, or more certainly by a higher rate of cannibalism by these females to
420 counterbalance the deficiency in dietary proteins and cover the high protein needs for
421 lactation. Indeed, protein restriction did not affect the litter size in rats (40) but did affect the
422 pup mortality rate due to maternal cannibalism (1).

423 Finally, diets also significantly affected the growth of pups. Pups from the high-lipid group
424 grew more slowly than those from the high-protein group, and did not catch up on their
425 growth rate during the 105 day sampling period. Reduced growth rate during suckling was
426 probably linked to a lack of protein in the milk. As shown in the literature, the milk

427 composition of females exposed to a low protein diet is modified by the reduction in protein
428 concentration and the increased lipid concentration, which reduces the growth rate of pups in
429 comparison with mothers eating an optimal protein diet (31).

430 We found that the growth rate of pups was significantly affected by diet, and that the effects
431 of the diet were sex and age specific. In other species such as rats (6), mice (9) and golden-
432 mantled ground squirrels (30), the growth rate of pups is also inversely related to litter size.
433 On the contrary, we found that the growth rate of pups from the high-protein group was
434 higher than that of pups from the high-lipid group, despite females of the high-protein group
435 having larger litters.

436

437 *Perspective and significance*

438 This study of the hibernation pattern, consecutive reproduction and growth rate of pups in the
439 European hamster demonstrates a strong impact of macronutrient diet composition and
440 particularly protein content on the annual lifecycle of the European hamster. To the best of
441 our knowledge, this is the first study demonstrating the crucial role of dietary proteins on both
442 hibernation and subsequent reproduction in a food-storing hibernator. We demonstrate a
443 plasticity in the responses of adults to diet composition in order to manage the hibernation
444 period. However, a low protein diet impaired reproductive success, which could affect
445 population dynamics in the wild. The impact of the two diets on reproduction was only
446 investigated in females. It would be interesting to conduct similar experiments in males, since
447 the duration of hibernation directly impacts their mating opportunities and thus also their
448 reproductive success (8). Additional studies of milk composition in the two groups could
449 provide more information on the influence of diet on milk composition. A study across
450 several hamster generations could make it possible to confirm these results on hibernation and
451 reproduction.

452 This new result opens a new avenue of research evaluating how diet composition impacts
453 sexual maturation in the juvenile hamster, which could, in turn, affect the reproduction
454 strategies of this species. The environmental conditions of this species have changed
455 drastically over the last 50 years, with an increase in agricultural plot size, the development of
456 mono-cropping and the progressive disappearance of soil micro-fauna (37). In this context,
457 our data provides new knowledge to enrich current conservation plans to improve the natural
458 agricultural habitat of this species in Europe and restore its population levels.

459

460

461 Ethics

462 The experimental protocols followed EU Directive 2010/63/EU guidelines for animal
463 experiments and the care and use of laboratory animals, and was approved by the Ethical
464 Committee (CREMEAS) under agreement no. 00624-01.

465

466 Conflicts of interest

467 On behalf of all authors, the corresponding author states that there is no conflict of interest.

468

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474

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477

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610

611

612

613 **Tables**

614

	high-protein diet	high-lipid diet
Energy (kJ/kg)	14503	15483
Carbohydrates	54.9 (63%)	57.0 (62%)
Lipids	5.1 (13%)	10.0 (24%)
Proteins	19.3 (23%)	12.8 (15%)

615

616 **Table 1. Energy value and composition of the diets.** Data are represented as a percentage of

617 fresh mass and a percentage of energy content (in brackets).

618

Diet group	high-protein	high-lipid
Number of torpor bouts (BT<32°C)	26.6 ± 0.6	22.6 ± 2.8 *
Mean duration of torpor bouts (BT<32°C) (hours)	90.5 ± 2.1	96.2 ± 11.6
Total time spent in torpor (BT<32°C) (hours)	2397.0 ± 37.0	2152.0 ± 257.0 *
Number of inter-torpor euthermic phases (BT>32°C)	25.4 ± 0.6	21.0 ± 1.0 **
Mean duration of inter-torpor euthermic phases (BT>32°C) (hours)	29.4 ± 1.9	19.3 ± 1.0 **
Total time spent in inter-torpor euthermic phases (BT>32°C) (hours)	747.0 ± 51.3	396.4 ± 41.0 ***

619 **Table 2. Diet effects on the hibernation behaviour of hamsters.** Results are presented as
 620 mean values \pm SEM. Asterisks indicate significant differences between high-protein and high-
 621 lipid groups (*: $p < 0.05$, **: $p < 0.005$, ***: $p = 0.001$).

622

Diet group	Number of mating events	Number of litters		Litter size	
		<i>Parturition</i>	<i>Weaning</i>	<i>Parturition</i>	<i>Weaning</i>
high-protein	32	2.0 \pm 0.2	1.9 \pm 0.2	6.5 \pm 1.0	5.8 \pm 1.2
high-lipid	37	1.7 \pm 0.2	1.2 \pm 0.2*	3.4 \pm 1.0 ***	1.9 \pm 1.2 ***

623 **Table 3. Numbers of mating events, average number of litters and average litter size at**
 624 **parturition and at weaning for each diet group.** Results are mean values \pm SEM. Asterisks
 625 indicate significant differences between values from a same column (*: $p < 0.05$, ***:
 626 $p = 0.001$).

627

628

629

630 **Figure legends:**

631

632 Fig. 1. A: Effects of the diets on the body mass and body composition of hamsters. Mean
633 body mass (g) for high-protein and high-lipid groups during the pre-hibernation and post-
634 hibernation phases. The hatched area depicts the hibernation phase. Results are represented as
635 mean values \pm SD. Asterisks indicate significant differences between high-protein and high-
636 lipid groups ($p < 0.05$); B: Mean fat mass (g) for high-protein and high-lipid groups before and
637 after hibernation, C: Mean fat-free mass (g) for high-protein and high-lipid groups with the
638 different compartments composing FFM before and after hibernation. Results are represented
639 as mean values \pm SEM. Different letters show significant differences in fat mass and fat-free
640 mass compartments (the 3 compartments having been analysed separately) between diets in
641 January and September ($p < 0.05$). Asterisks indicate significant differences in fat-free mass
642 between diets in January and September ($p < 0.05$).

643

644 Fig. 2. Average daily intake of food (A), energy (B), protein (C), lipid (D), SFA (E) and
645 PUFA (F) according to the month (pre-hibernation period) and the diet group (high-protein
646 and high-lipid). Data are represented in $\text{kJ}\cdot\text{day}^{-1}$ for panel B and in $\text{g}\cdot\text{day}^{-1}$ in the remaining
647 panels; asterisks indicate significant differences between the diet groups. Results are
648 represented as mean values \pm SEM.

649

650 Fig. 3. Changes in the body mass of pups according to sex and diet from the age of seven to
651 105 days. Females are shown in black and males in grey. Circles and triangles represent data
652 for the high-lipid and high-protein group, respectively. Different letters indicate significant

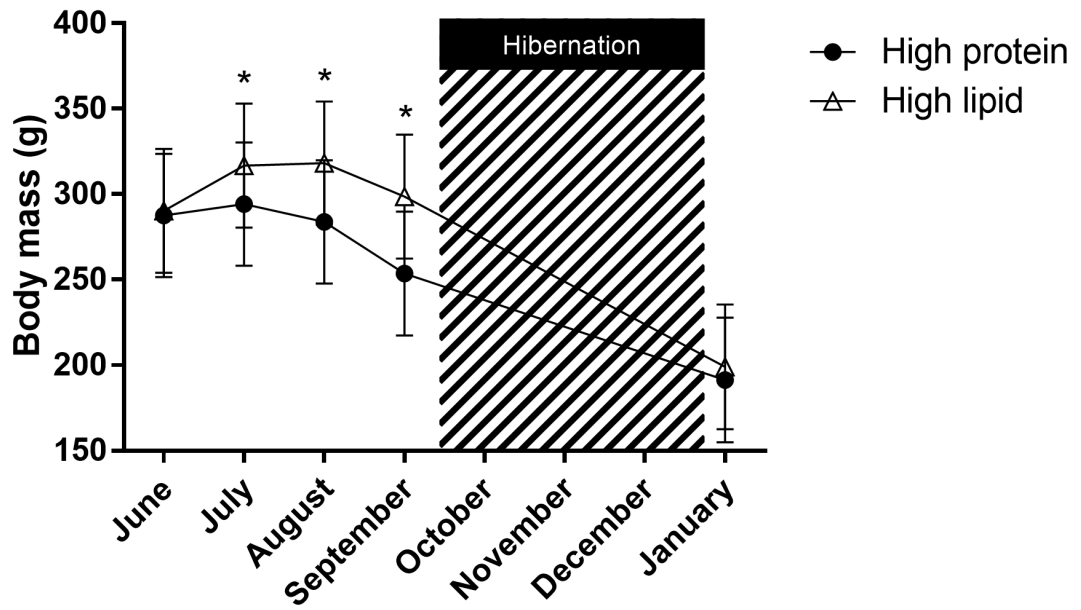
653 differences ($p < 0.05$) between groups (see Results section for more details). Results are
654 represented as mean values \pm SEM. The dashed line represents the weaning date.

655

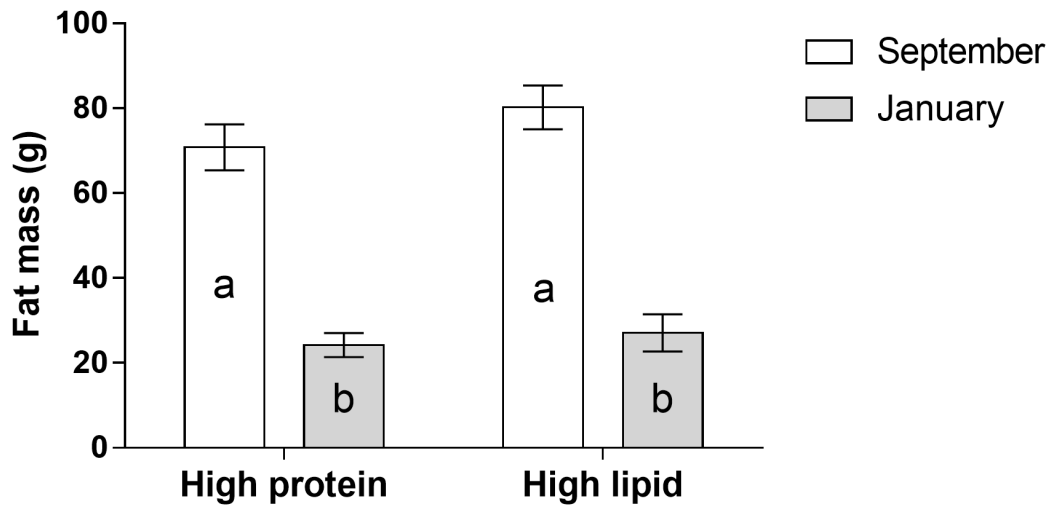
656 Fig 4. Proposed model of how the two diets affected the hibernation behaviour and
657 reproductive performances of European hamsters. The high-lipid group (shown in orange) had
658 a higher body mass before hibernation than the high-protein group (shown in blue). The high-
659 lipid group spent less time in torpor and thus lost more body mass during hibernation than the
660 high-protein group. Despite similar body conditions in both groups at the start of
661 reproduction, the high-protein group had a higher reproductive success with bigger litters and
662 larger pups.

Figure 1

A



B



C

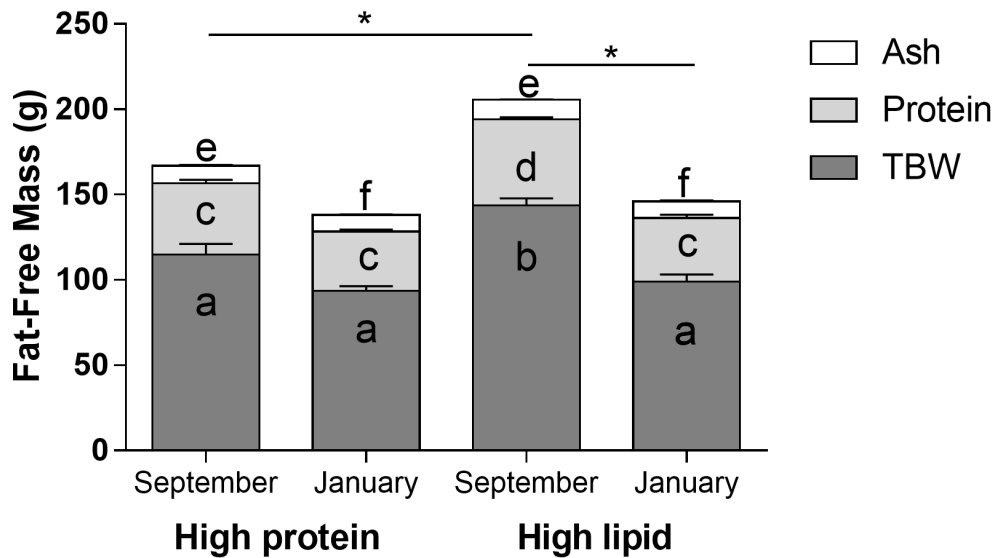


Figure 2

