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1 Dietary proteins improve hibernation and subsequent reproduction  
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3

4 Mathieu Weitten<sup>1</sup>, Mathilde Louise Tissier<sup>1</sup>, Jean-Patrice Robin<sup>1</sup>, Caroline Habold<sup>1</sup>✉

5 <sup>1</sup>Université de Strasbourg, CNRS, IPHC UMR 7178, F-67000 Strasbourg, France

6 ✉ Corresponding author: [caroline.habold@iphc.cnrs.fr](mailto:caroline.habold@iphc.cnrs.fr)

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 4<sup>th</sup>  
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 =  $286.9 \pm 6.3$ 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 ( $\pm 0.1$ g) and  
132 calculating the difference between the amount of pellets they received and the amount  
133 remaining. Animals were weighed monthly ( $\pm 0.1$ 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  $3.2 \pm 0.1$ 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  $32^\circ\text{C}$  (29).

146 *C. Body composition assessment*

147 After euthanasia by decapitation under 2% isoflurane anaesthesia, the carcass was weighed  
148 ( $\pm 0.01$ 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 ( $\pm 0.1$  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.*  $-80.9\pm 4.1$  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 =  $287.7\pm 5.1$  g,  
225 high-lipid group =  $290.3\pm 5.3$  g,  $p = 0.73$ ) and at the end of this first experiment (January,  
226 high-protein group =  $187.7\pm 9.5$  g, high-lipid group =  $196.5\pm 9.5$  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  $22.6\pm 6.1$  g in July,  $34.8\pm 6.5$  g in August and  $42.2\pm 11.6$  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  $2.76\pm 0.02$  and high-lipid group =  $2.84\pm 0.04$ , 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  $2.84\pm 0.04$   
243 to  $2.66\pm 0.04$  (Wilcoxon test:  $p=0.01$ ), whereas no change was observed in the high-protein  
244 group (ranging between  $2.76\pm 0.02$  and  $2.68\pm 0.04$ ). 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 ( $7.4\pm 1.0$  g.day<sup>-1</sup>) than individuals in the high-lipid group  
253 ( $8.3\pm 1.0$  g.day<sup>-1</sup>; mean difference =  $-0.92\pm 0.40$  g.day<sup>-1</sup>). All females in both groups ingested  
254 on average more food in June and July ( $8.7\pm 1.0$  g.day<sup>-1</sup> in June and  $8.3\pm 1.0$  g.day<sup>-1</sup> in July, no  
255 significant differences:  $p = 0.12$ ) than in August and September ( $7.5\pm 1.0$  g.day<sup>-1</sup> in August  
256 and  $6.8\pm 1.0$  g.day<sup>-1</sup> 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  $8.2\pm 1.0$  g.day<sup>-1</sup> and high-lipid group =  $9.2\pm 1.0$  g.day<sup>-1</sup>), 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  $5.9\pm 1.1$  g.day<sup>-1</sup> in September), whereas it decreased  
262 by only  $\sim 15\%$  in the high-lipid group (reaching  $7.8\pm 1.1$  g.day<sup>-1</sup> 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 =  $-0.184 \pm 0.011$  g.day<sup>-1</sup>), and with a significant decrease in SFA  
295 daily intake from June to September (mean difference =  $-0.031 \pm 0.007$  g.day<sup>-1</sup>,  $p < 0.001$ ). 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 =  $-0.291 \pm 0.026$  g.day<sup>-1</sup>) and showed a decrease in the PUFA  
300 intake from June to September (mean difference =  $-0.093 \pm 0.017$  g.day<sup>-1</sup>,  $p < 0.001$ ). 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 =  $-0.33 \pm 0.05$  g.day<sup>-1</sup>,  $p < 0.001$ , compared to August (mean difference =  $-0.30 \pm 0.03$   
305 g.day<sup>-1</sup>,  $p < 0.001$ ), June (mean difference =  $-0.29 \pm 0.03$  g.day<sup>-1</sup>,  $p < 0.001$ ) and July (mean  
306 difference =  $-0.24 \pm 0.02$  g.day<sup>-1</sup>,  $p < 0.001$ )].

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 =  $7.38 \pm 0.04$ , 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 ( $3144.3 \pm 77.5$  hours for high-protein group and  $2548.2 \pm 99.2$  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,  $\text{Rho}=-0.845$ ,  $p<0.001$ ).

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:  $4.9 \pm 0.9$  and second litter:  $5.1 \pm 1.0$  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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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

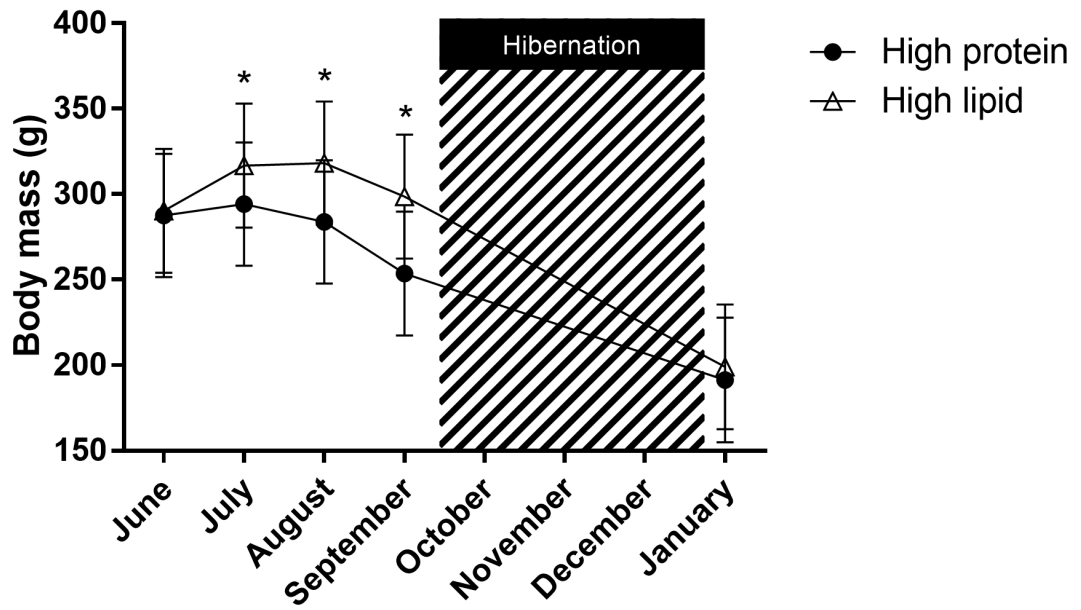
653 differences ( $p < 0.05$ ) between groups (see Results section for more details). Results are  
654 represented as mean values  $\pm$  SEM. The dashed line represents the weaning date.

655

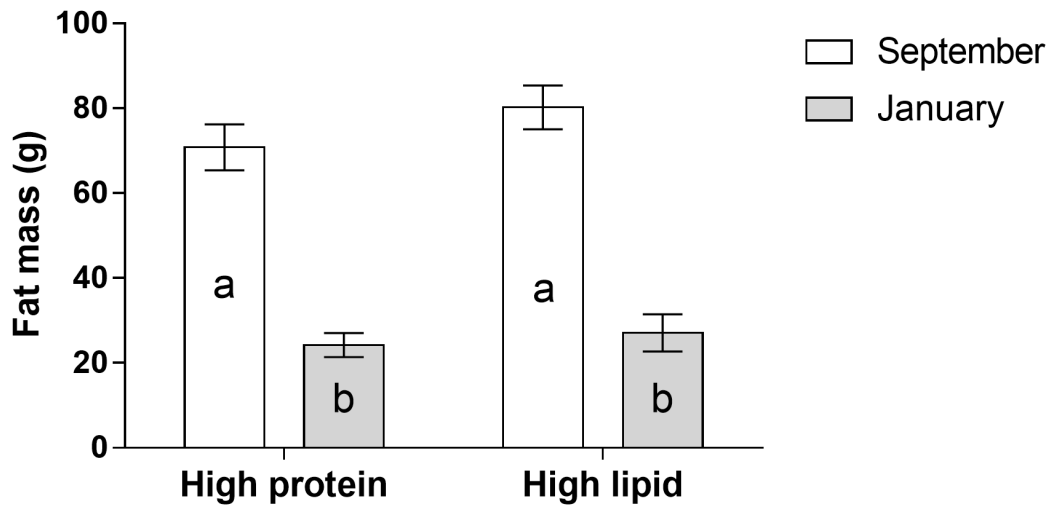
656 Fig 4. Proposed model of how the two diets affected the hibernation behaviour and  
657 reproductive performances of European hamsters. The high-lipid group (shown in orange) had  
658 a higher body mass before hibernation than the high-protein group (shown in blue). The high-  
659 lipid group spent 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**

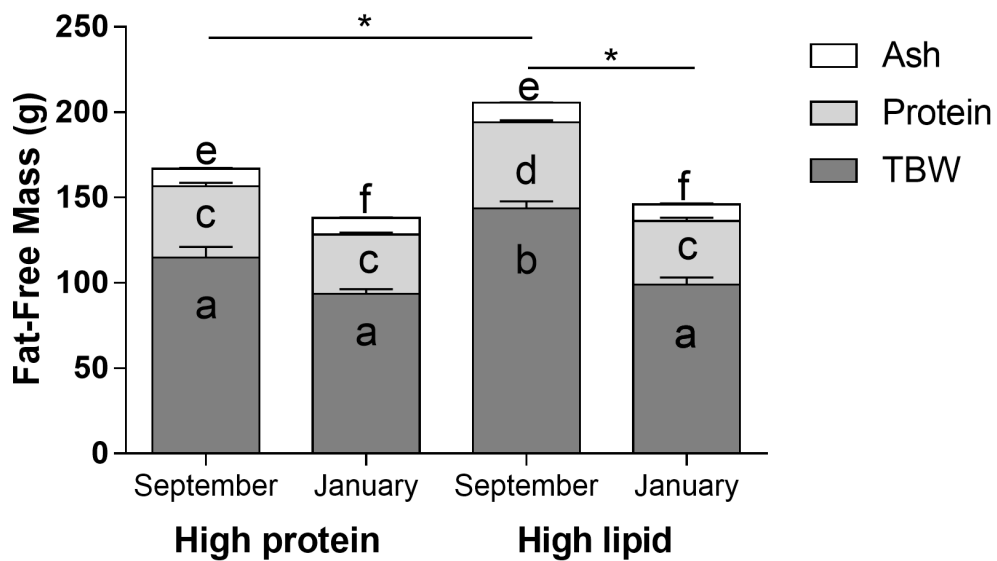




Figure 2

