

1 *Running title:* Energy budget and hibernation in a marsupial

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3 **A mesocosm experiment in ecological physiology: adaptive modulation of energy**
4 **budget in a hibernating marsupial under chronic caloric restriction**

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6 Roberto F. Nespolo^{1,2,3*}, Francisco E. Fontúrbel⁴, Carlos Mejias², Rodrigo Contreras²,
7 Paulina Gutierrez², José Ruiz², Esteban Oda², Pablo Sabat⁵, Catherine Hambly⁶, John R.
8 Speakman^{6,7,8} & Francisco Bozinovic¹

9

10 ¹ Center of Applied Ecology and Sustainability (CAPES), Departamento de Ecología
11 Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

12 ² Instituto de Ciencias Ambientales y Evolutivas, Universidad Austral de Chile, Valdivia,
13 Chile

14 ³ Millennium Institute for Integrative Biology (iBio), Santiago, Chile

15 ⁴ Instituto de Biología, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile.
16 ORCID 0000-0001-8585-2816

17 ⁵ Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile,
18 Santiago, Chile. ORCID 0000-0002-6609-9969

19 ⁶ Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen,
20 AB24 2TZ, UK

21 ⁷ State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and
22 Developmental Biology, Chinese Academy of Sciences, Beijing, 100101, China

23 ⁸ Chinese Academy of Sciences Center of Excellence in Animal Evolution and Genetics,
24 Kunming, China

25 * *Corresponding author:* robertonespolo@uach.cl

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35 acceptance.

36 **Abstract**

37 During the last sixty years, mammalian hibernation (i.e., seasonal torpor) has been interpreted
38 as a physiological adaptation for energy economy. However -and crucially for validating this
39 idea - direct field comparisons of energy expenditure in hibernating and active free-ranging
40 animals are scarce. Using replicated mesocosms and a combination of energy budgeting
41 approaches (i.e., doubly labelled water, rates of CO₂ production and food intake), we
42 experimentally manipulated energy availability and quantified net energy costs of
43 hibernation in a marsupial. We hypothesized that, when facing chronic caloric restriction
44 (CCR), a hibernator should maximize torpor use for compensating the energetic deficit,
45 compared to *ad libitum* fed individuals (=controls). However, intensifying torpor duration at
46 low temperatures could increase other burdens (e.g., cost of rewarming, freezing risk). In
47 order to explore this trade-off, we followed the complete hibernation cycle of the relict
48 marsupial *Dromiciops gliroides*, and estimated its total energy requirements, and compared
49 this with a control condition. Our results revealed: (1) that energy restricted animals, instead
50 of promoting heat conservation strategies during hibernation (e.g., social clustering and
51 thermoregulation), maximized torpor use and saved just enough energy to cover the deficit,
52 and (2) that hibernation represents a net energy saving of 51% compared with animals that
53 remained active. This work provides compelling evidence of a fine-tuning use of hibernation
54 in response to food availability and presents the first direct estimation of energy savings by
55 hibernation encompassing the total hibernation cycle.

56

57 **Key words:** behavioral thermoregulation, chronic caloric restriction, daily energy
58 expenditure, doubly labelled water, energy budget, hibernation, marsupial, social
59 clustering.

60 **Introduction**

61 The countless ways natural selection shapes organismal design and function has always
62 intrigued biologists, particularly in ecosystems where energy availability is diluted,
63 temporally or spatially variable (Mueller and Diamond 2001, Ferguson 2002, Nie et al. 2015).
64 In this scenario, energy flow is often explained by the allocation principle, where energy
65 from food passes through several sequential bottlenecks (e.g., foraging, digestion,
66 assimilation), and must be allocated to different functions in parallel (e.g., growth,
67 maintenance, and reproduction) (Weiner 1992). From this perspective, nature's economy
68 would be defined by austerity, for which ectotherms provide the best fit to the rule, as they
69 minimize maintenance costs when activity is low (Pough 1980, Artacho and Nespolo 2009).
70 Endotherms (birds and mammals) on the opposite have a wasteful lifestyle, a counter-
71 intuitive solution for any idea of nature's economy (an "extravagant economy" *sensu* (Hayes
72 and Garland 1995, Koteja 2004). However, some endotherms experience transient periods of
73 ectothermy or torpor (=heterothermy, hereafter), as putative adaptations to seasonal or
74 unpredictable reductions in environmental productivity. For the case of hibernation (i.e.,
75 seasonal multi-day episodes of torpor)(Geiser and Ruf 1995b), animals experience drops in
76 body temperature and a general reduction in metabolism lasting several days or weeks, where
77 body temperature is maintained a few degrees above ambient temperature. During these
78 episodes, maintenance costs fall to a fraction of normal values, with significant energy
79 savings (Geiser 2004), a "logical" solution for animals that cannot migrate to better
80 environments (Schmidt-Nielsen 1979). Thus, hibernators would have the long-term benefits
81 of endothermy, together with the short-term benefits of ectothermy.

82 Contrarily with daily torpor, where metabolic depression occurs during a few hours,
83 hibernation is characterized by torpor events that increase in duration and frequency as the
84 cold season progresses {Geiser, 2013 #10429}. Thus, animals modulate the frequency of
85 such events depending on the cold, photoperiod and the amount of fat reserves, the latter
86 being determinant on predicting hibernation survival (Humphries et al. 2002, Humphries et
87 al. 2003a, Humphries et al. 2003b). But how much energy, exactly, is saved during a
88 complete hibernation cycle, compared to a situation without hibernation? Do hibernating
89 animals regulate torpor frequency "wisely" as food availability varies? Although hundreds
90 of laboratory experiments have provided partial answers to these questions, only a handful

91 of experimental manipulations of food availability have demonstrated a link between energy
92 availability, torpor frequency and fat reserves in hibernation (reviewed in (Vuarin and Henry
93 2014).

94 According to Boyles et al. (Boyles et al. 2020), to compensate for reduced energy
95 availability, a hibernator that perceives an energetic bottleneck in the environment should
96 experience longer and deeper torpor bouts and select sites with low temperatures for
97 hibernating (Song et al. 2000). However, this has a limit imposed by several costs (e.g.,
98 prolonged inactivity, freezing mortality, decreasing immune function and sleep deprivation,
99 see Humphries et al. 2003b, Boyles et al. 2020), which furnishes a “hibernation trade-off”
100 where an optimum (minimum) hibernation temperature is defined {Humphries, 2002
101 #10368}. Above this temperature, energy saved by hibernation is maximized and below this
102 temperature, hibernation costs are maximized. In nature, a range of responses have been
103 observed. For instance, passerine birds (Wojciechowski et al. 2011, Douglas et al. 2017),
104 mice (Eto et al. 2015) and Siberian hamsters (Jefimow et al. 2011) minimize heat loss during
105 daily torpor, whereas non-migrating bats (Ryan et al. 2019) and sugar gliders (Nowack and
106 Geiser 2016) minimize body temperature during multi-day hibernation.

107 Here we explored the hibernation trade-off on the social Microbiotheriid marsupial
108 *Dromiciops gliroides* (Hershkovitz 1999) using a mesocosm setup for tracking animals
109 during a complete hibernation cycle. Specifically, we manipulated food by applying a chronic
110 caloric restriction treatment (CCR) and we measured total energy requirements for wintering
111 using gross energy intake (=daily food consumption) and CO₂ production, using the doubly
112 labelled method. Specifically, we predicted that CCR animals (compared with ad libitum fed
113 animals) will either intensify torpor use in order to maximize energy savings and compensate
114 for the energy restriction they will avoid risks by using heat conservation strategies (e.g.,
115 social clustering and hibernacula use).

116

117 **Methods**

118 *Animals*

119 *Dromiciops gliroides* (Thomas 1894) is the only living species of Microbiotheria; the
120 ancestral group of Australian marsupials. *D. gliroides* is a small arboreal marsupial inhabiting
121 the temperate rainforests of southern South America, living in native forest stands dominated

122 by *Nothofagus* spp. and *Araucaria araucana* trees (HersHKovitz 1999, Fonturbel et al. 2012).
123 This marsupial is known to be the sole disperser of several endemic plant species, thus being
124 intimately associated with the temperate rainforest (Amico et al. 2009), where this
125 experiment was performed. We installed the mesocosm in Estación Experimental Fundo San
126 Martín (SM), a property of Universidad Austral de Chile (39° 41'S 73° 18'W), which is
127 within the typical habitat of *Dromiciops gliroides*. In this paper we refer to “hibernation” as
128 the multiday torpor bouts lasting several days, in contrast to daily heterotherms that
129 experiences torpor bouts of 3-12 hours (Geiser and Ruf 1995a). No previous monitoring of
130 the whole hibernation period of *D. gliroides* is available, which was estimated to extend from
131 May to September (HersHKovitz 1999, Muñoz-Pedrerros et al. 2005). Thus, we started the
132 experiment in April, and finished data gathering in December. We captured 40 individuals
133 from different sites within SM during the austral summer, which were live-captured
134 using Tomahawk-like traps baited with banana and attached to the trees, 2 m above the
135 ground (Fonturbel 2010). Traps were located 300 m apart from the enclosure site, in four
136 different patches of forest, each on a sampling grid. Each individual was marked using PIT-
137 tag (BTS-ID, Sweden) subcutaneous mark, and transported to the laboratory immediately
138 after capture for feeding and rehydration.

139

140 *Outdoor enclosures*

141 To characterize simultaneously physiological and thermoregulatory responses of
142 hibernating *D. gliroides*, we built eight cylindric enclosures (Fig. 1), which were distributed
143 within the forest and separated about 5 m from each other, covering a total area of about 80
144 m² (see Supplementary Material). Each enclosure had a internal volume of 2 m³, and was
145 manufactured in zinc with a large 1.8m-diameter cylinder buried 10 cm in the ground, which
146 gave a 0.8 m height above ground. Each enclosure had a data logger installed for continuous
147 measurement of air temperature (HOBO ®). Initially, four enclosures were assigned to a
148 control treatment (“control”, hereafter) and the other four were assigned to a caloric
149 restriction treatment (“CCR”, hereafter; see below). Five unrelated animals (i.e., from
150 different sites to avoid kinship effects) (Franco et al. 2011) were released in each enclosure,
151 on April 1st (autumn). Unfortunately, one of the CCR enclosures was destroyed by a tree

152 falling during winter (animals escaped), which left us with an unbalanced design with 35
153 animals across 7 enclosures (4 controls and 3 CCR).

154

155 *Experimental energy manipulation*

156 To explore how constant food shortage induce compensatory responses during
157 hibernation, we applied a chronic caloric restriction treatment to three enclosures. Then, we
158 offered the equivalent of 165 kJ ind⁻¹d⁻¹ for the control enclosures and provided to the CCR
159 animals, 60% of this value (95 kJ ind⁻¹d⁻¹). The food was provided in equal volumes every
160 day, but once a week we provided a fresh weighed amount (± 0.01 g) to each enclosure and
161 weighed the fresh weight of the leftovers for drying to constant weight (60°C). With this, we
162 estimated the water content of the diets for estimating average energy intake. Using weekly
163 values of energy consumption, we calculated the (per capita) total hibernation energy
164 requirements (kJ per individual).

165

166 *Torpor thermoregulation and daily energy expenditure*

167 Weekly, we took digital thermographic images to clustered torpid individuals in order to
168 estimate the thermal differential between animals and substrate and to relate this to the caloric
169 restriction treatment (Fig 1a). We also recorded cluster sizes and whether animals were
170 within or outside the hibernaculum (see Supplementary Material). To determine direct daily
171 energy expenditure (DEE, kJ/day), we applied the doubly labelled water technique (Lifson
172 and McClintock 1966, Butler et al. 2004)(see Supplementary Material) on 24 captive
173 individuals before the release on enclosures (week zero, in summer, indicated in Fig 2a), we
174 successfully repeated these determinations in 16 animals at week 18 of the experiment (late
175 winter; eight individuals from the CCR treatment and eight from the control treatment), thus
176 giving an average DEE for 48 hours. Basal metabolic rate (BMR) was determined from the
177 rate of CO₂ production in these same animals measured in the laboratory using standard
178 respirometry techniques (Nespolo et al. 2010, Contreras et al. 2014).

179

180 *Statistical analysis*

181 We used a combination of generalized linear mixed models and standard parametric analyses
182 such as ANCOVA, ANOVA and linear regressions when justified. Detailed descriptions of
183 statistical analyses are provided in Supplementary Material.

184 All procedures presented in this study were approved by the Chilean Agriculture and
185 Livestock Bureau (SAG) permits No 4371/2019 and 3393/2019, and by the Bioethics
186 Committee of the Austral University of Chile, resolution 313/2018 annex 2019.

187

188 **Results**

189 The main outcome of this experiment supports the idea that hibernating *D. gliroides*
190 modulated torpor use for saving energy and cover the energetic deficit imposed by caloric
191 restriction (results summarized in Fig. 2 and Supplementary Table 2). Indeed, animals under
192 CCR (n= 15) consumed similar amounts of food as controls initially, but approximately at
193 the eleventh week they consumed significantly less food than controls (n=20)(Fig 2b). CCR
194 animals did not prefer to cluster in larger groups or use hibernacula for heat maintenance,
195 and no statistical differences in any thermoregulatory aspect of the comparison of CCR and
196 controls groups were observed (see Figs S2 and S3 in Supplementary Material). Moreover,
197 those individuals experienced a constant reduction of body mass (M_B); to become significant
198 at the 20th week (Fig. 2a). At week 23, however, CCR animals started to recover M_B and
199 were not significantly different from controls by week 25 (two-tailed t-tests, $p < 0.001$; Fig.
200 2a), thus suggesting that they, without access to extra food, managed their energy budget
201 more efficiently. This is confirmed by measurements of per-capita energy consumption,
202 which shows CCR animals consistently ingested less food than controls, until the rise in
203 ambient temperatures during the austral spring (Fig. 2b-c). Then, energy intake became
204 significantly higher in control individuals compared to CCR individuals at week 10 until
205 week 24 (two-tailed t-tests, $p < 0.01$; Fig 2b). This is explained by a higher incidence in
206 torpor use in CCR animals compared to controls, a difference that was the largest during
207 August, which suggests that the main trigger of torpor was body condition rather than
208 immediate food availability (Fig. 2c). Control animals attained a maximum weight loss of
209 $13.2 \pm 5.1\%$ (mean \pm sem) by week 19, whereas CCR animals reached a weigh loss of 34.8
210 $\pm 3.1\%$ by week 20. Also, daily energy intake was significantly correlated with air
211 temperature in CCR animals ($p < 0.01$, $n=432$) whereas this correlation was non-significant

212 for control animals ($p=0.08$, $n=652$; Fig 3). Thus, although CCR animals had access to 95 kJ
213 ind^{-1} per day, they reduced energy consumption to about half of this value ($=47.7 \pm 3.9$ kJ
214 $\text{day}^{-1} \text{ind}^{-1}$, week 8-18, $n=3$ enclosures), which was significantly lower than that in controls
215 (96.7 ± 7.3 kJ $\text{day}^{-1} \text{ind}^{-1}$, week 8-18, $n=4$ enclosures) ($p \ll 0.001$, t-test). This allowed them
216 to reduce total winter energy requirements (i.e., per capita, E_w) to 46% of the controls
217 (control: $E_w = 10,066 \pm 593.9$ kJ ind^{-1} , $n=4$ enclosures; CCR: $E_w = 4,583.8 \pm 113.6$ kJ ind^{-1} ,
218 $n=3$ enclosures; $p \ll 0.001$, t-test).

219 During the winter period (i.e., between weeks 8 to 18), animals exhibited an
220 approximately constant negative slope in body mass (see Fig 2a). On average, each animal
221 lost 3.0g (control) and 5.5g (CCR) in 70 days (i.e., 0.042 and 0.079 g $\text{day}^{-1} \text{ind}^{-1}$, respectively),
222 which can be assumed to be 60% body fat (Mitchell et al. 2015). Thus, with an energy content
223 of 39.7 kJ g^{-1} for fat (Walsberg and Wolf 1995), this gives 1.0 and 1.9 kJ $\text{day}^{-1} \text{ind}^{-1}$, for each
224 condition respectively. Thus, daily energy expenditure from food and body fat consumption
225 can then be calculated as $DEE = E_w + E_{FAT}$ in each case (being $DEE_{CONTROL} = E_{w-control} +$
226 $E_{FATcontrol}$ and $DEE_{CCR} = E_{w-ccr} + E_{FATccr}$). This gives: $DEE_{CONTROL} = 98.6$ kJ $\text{day}^{-1} \text{ind}^{-1}$ and
227 $DEE_{CCR} = 48.7$ kJ $\text{day}^{-1} \text{ind}^{-1}$. Thus, control animals, which were active at the moment of
228 sampling, spent on average twice the amount of energy of CCR animals, which were in deep
229 torpor.

230 The doubly labelled water measurements show that summer animals had a DEE of
231 44.9 ± 2.2 kJ $\text{day}^{-1} \text{ind}^{-1}$ ($n=24$) which is 58% of the expected DEE for mammals (Nagy 2005).
232 This increased significantly in winter to 47.3 ± 5.6 kJ $\text{day}^{-1} \text{ind}^{-1}$ ($n=8$) (82% of the expected
233 value) in CCR animals and 88.0 ± 5.8 kJ $\text{day}^{-1} \text{ind}^{-1}$ ($n=8$) in controls (117% of the expected
234 value) ($F_{1,11}=8.92$, $P=0.012$, ANCOVA)(Fig 4a). There were no significant differences in
235 basal metabolic rate (BMR) across seasons and treatments (Fig 4b), but the factorial scope
236 for DEE (DEE/BMR), a measure of the aerobic work capacity, resulted significantly different
237 across seasons and treatments, where in winter control animals had 62% higher value
238 compared with CCR animals (6.45 ± 0.58 over 4.04 ± 0.45 , Fig 4c; $F_{1,11}=5.37$, $P=0.040$,
239 ANOVA)(Fig 4c). Body mass was significantly reduced in CCR animals by 70% during
240 winter compared with their summer values, whereas control individuals did not show
241 seasonal differences (Fig 4d). Summer (pooled: control and CCRs) DEEs were significantly
242 correlated with body mass ($R^2=0.61$, $P=0.039$, $n=24$, Fig 4d-e), which was maintained in

243 winter, with a difference in intercepts between control and CCR animals (Fig 4f, $F_{1,13}=8.32$,
244 $P=0.013$, ANCOVA).

245

246 **Discussion**

247 Several authors have calculated the amount of energy saved by specific sections of
248 the hibernation cycle, frequently in a single torpor-arousal cycle and sometimes during
249 multiple events (Geiser 1988, Holloway and Geiser 1995, Schmid and Speakman 2000,
250 Bozinovic et al. 2007, Nespolo et al. 2010, Geiser 2013). These values vary from 99% in
251 single torpor bouts compared with normothermic values, to 15% for multi-day torpor bouts
252 in some hibernators, including the costs of arousals (Wang 1978, Geiser 2004, 2013).
253 However, establishing the precise impact of hibernation on the energy budget of free ranging
254 animals is especially difficult, since a control condition (i.e., a situation without hibernation,
255 keeping all else equal) is hard to obtain. To the best of our knowledge, this has been
256 calculated indirectly on laboratory animals, once in eutherians, the Richardson's ground
257 squirrel (*Urocitellus richardsonii*, (Wang 1978) and once in a marsupial, in pygmy-possum
258 (*Cercartetus nannus*; (Geiser 2007). Both estimations indicate enormous energy savings by
259 hibernation: 87.7% and 97.5%, respectively, after comparing hibernation energy expenditure
260 with the predicted metabolism of active animals. Our results of daily energy expenditure
261 (DEE) in energy restricted animals and controls provide a direct estimation of this value, with
262 the caveat that during the coldest months (July-October) on average only 69% of CCR
263 animals were in torpor and 25% of controls were in a similar condition. However, these
264 values coincide well with the doubly labelled water method (DLW) estimations, for which
265 all CCR animals were torpid at the moment of sampling, and all control animals were active
266 at this moment. Recalling from Results, $DEE_{CONTROL-FOOD} = 98.6 \text{ kJ day}^{-1}\text{ind}^{-1}$ and
267 $DEE_{CONTROL-DLW} = 88.0 \text{ kJ day}^{-1}\text{ind}^{-1}$, and averaging, gives $DEE_{CONTROL} = 93.3 \text{ kJ day}^{-1}\text{ind}^{-1}$.
268 On the other hand, $DEE_{CCR-FOOD} = 48.7 \text{ kJ day}^{-1}\text{ind}^{-1}$ and $DEE_{CCR-DLW} = 47.3 \text{ kJ day}^{-1}\text{ind}^{-1}$,
269 gives an average $DEE_{HIBERNATION} = 48.0 \text{ kJ day}^{-1}\text{ind}^{-1}$. This reveals a net hibernation
270 savings of 51.4% ($=DEE_{HIBERNATION} / DEE_{CONTROL}$). This smaller value, compared with
271 Belding's ground squirrel and pigmy possums can be explained by the fact that our
272 *Dromiciops* were experiencing outdoor/field conditions, which includes the thermal impact
273 of natural thermal variations and spontaneous activity bursts during interbout arousals.

274 According to Humphries et al. (2002)(Humphries et al. 2002) (see also: (French
275 1985), fat reserves predict wintering hibernation survival, because when “the size of the
276 reserve is less than the rate of depletion times the length of the winter, the hibernator will not
277 survive”. This assertion is true assuming that animals don’t ingest food during hibernation
278 (but see Fig 3). Without eating, a hibernating *D. gliroides* spending $48 \text{ kJ day}^{-1} \text{ ind}^{-1}$ will
279 need 4,320 grams of fat to survive a winter of 90 days (energy content of fat: 39.7 kJg^{-1})
280 ¹(Walsberg and Wolf 1995), which is unrealistic for a 40g animal. It is clear then, that
281 animals regulate food ingestion during interbout arousals, in some way “calculating” torpor
282 incidence for energy management.

283 Basal metabolic rate (BMR), which is one of the most measured variable in
284 physiological ecology, representing maintenance costs in endotherms (Konarzewski and
285 Diamond 1995, Ricklefs et al. 1996, White and Seymour 2003, McKechnie et al. 2006,
286 Clarke et al. 2010), surprisingly did not vary between seasons or treatments. Instead, the
287 scope for aerobic activity (DEE/BMR), a measure of how hard animals are working when
288 active, showed a significant 89% increase from summer to winter in control animals, but a
289 modest 37.9% increase in CCR animals (from Fig 4d). Thus, CCR animals, in addition of
290 saving energy by hibernation maintained a lower aerobic capacity probably by reducing the
291 amount of metabolically active tissues (Bozinovic et al. 1990, Campbell and MacArthur
292 1998, Nespolo et al. 2002).

293 Mueller and Diamond (2001)(Mueller and Diamond 2001) postulated food
294 availability (or net primary productivity) as a unifying factor for explaining adaptive
295 variation in energy expenditure across species, ecosystems, latitude, temperature or rainfall.
296 This idea is related to the more general “pace-of-life” theory of metabolism and life histories,
297 which proposes that populations evolving for a long time at low productivity also evolve low
298 levels of energy expenditure (Wikelski et al. 2003, Careau et al. 2010, Le Galliard et al. 2013,
299 Londono et al. 2015, Pettersen et al. 2016). Our results support the idea that hibernation
300 represents a “pace-of-life” adaptation to environments characterized by seasonal reductions
301 of primary productivity (i.e., characteristics of temperate regions), where hibernation acts a
302 physiologically regulated metabolic switch-off coupled with the period of low primary
303 productivity (winter){Turbill, 2011 #3341}. In this sense, the fact that hibernation is present
304 in several unrelated species living in the same environments supports the view of hibernation

305 as a convergent feature of mammals (Boyles et al. 2013). In fact, *D. gliroides*, the only South
306 American (SA) mammal described as a hibernator (Bozinovic et al. 2004), has a distribution
307 range in South America between 35° and 45° S, a narrow latitudinal strip that in the Southern
308 hemisphere includes a few landmasses (the tip of South Africa, Southern Australia including
309 Tasmania and most part of New Zealand). This contrasts with the vast extensions of
310 territories included in this range at the Northern hemisphere, from which almost all
311 hibernating species have been identified (Humphries et al. 2002, Boyles et al. 2008, Ruf and
312 Geiser 2015). Perhaps the right terrestrial environment at the Southern hemisphere simply
313 did not provide enough land area for hibernation to evolve more frequently.

314 Mesocosm studies (i.e., outdoor experiments examining natural environments under
315 controlled conditions) provide a fundamental link between field surveys and laboratory
316 experiments (Kennedy 1995, Verdier et al. 2014, Kurz et al. 2017, Maugendre et al. 2017,
317 Scharfenberger et al. 2019). However, they are particularly scarce in ecological physiology
318 (however, see references (Merritt et al. 2001, Levy et al. 2012, Gao et al. 2015), a field with
319 a long tradition on laboratory work (see ref (Humphries et al. 2003b) and cited references).
320 We encourage more of such experiments. Researchers will surprise how simple and cost-
321 effective they are, as one single long-term experiment could replace many small laboratory
322 trials.

323

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- 590

591 **Figure Captions**

592

593 Fig 1a) Digital photographs and thermographs of clustered hibernating *D. gliroides*, at
594 different cluster sizes. The average temperature of each picture is 10°C, approximately. b)
595 Photographs of the enclosures (c), enclosure opening showing the reproduced forest
596 environment, (d) female *Dromiciops* within the enclosure, (e) a male with the food feeders,
597 (f) a cluster of hibernating animals after removing the hibernaculum, (g) a close-up of a
598 cluster of 5 hibernating animals (h) a torpid female of the control treatment. Red arrows
599 indicate the moment of daily energy expenditure and basal metabolic rate measurements.

600

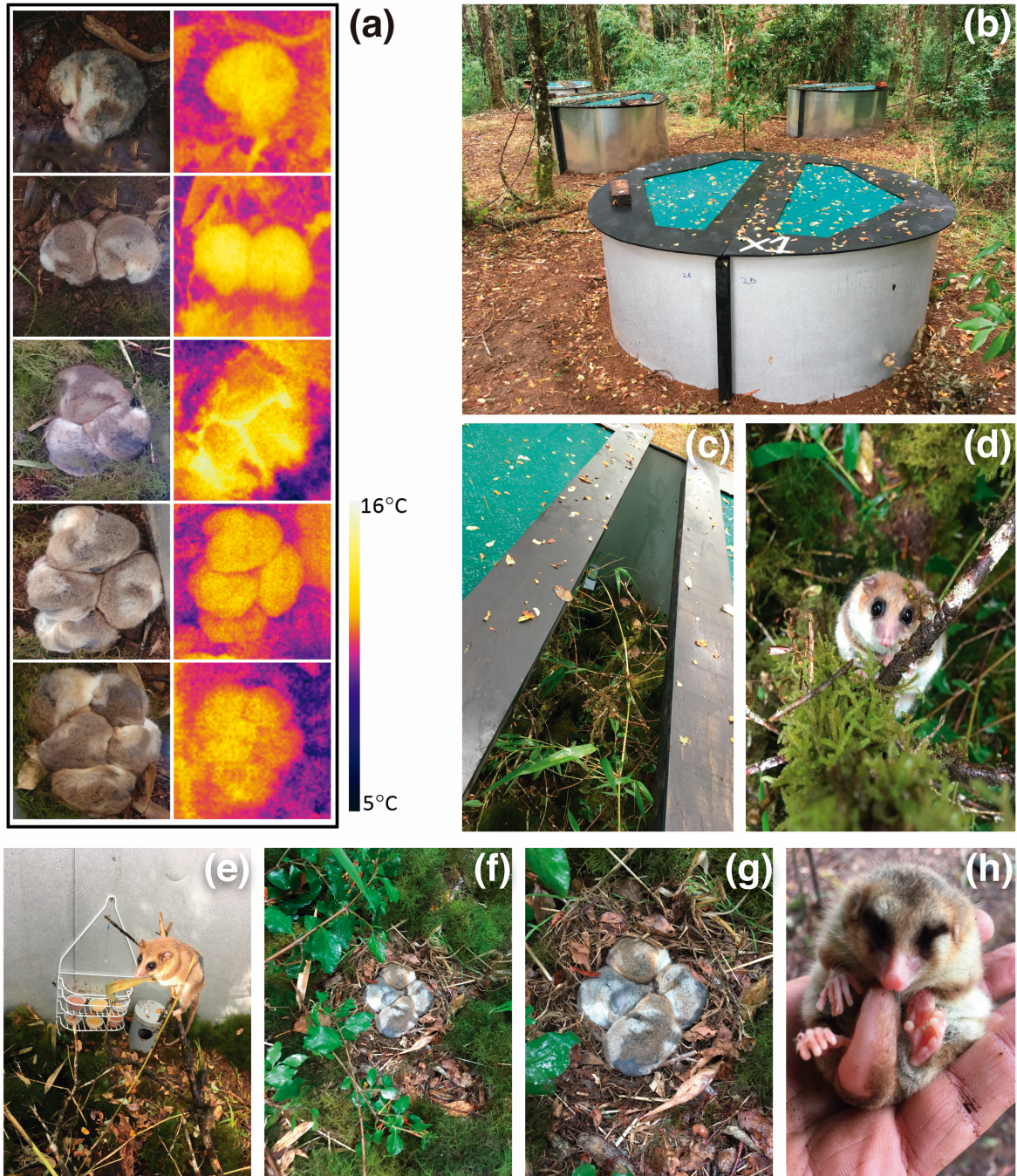
601 Fig 2. a) Weekly body masses (mean±sem) of individuals of *D. gliroides* either receiving
602 food ad libitum or exposed daily to a chronic energetic restriction, CCR, since week 0 (April,
603 15th, autumn), in a semi-natural experiment (enclosures). Comparisons between CCR (n=15)
604 and control (n=22) individuals were significant between week 20 and week 25 (t-tests,
605 $p<0.05$); b) Per-capita energy consumption (dry mass) showing control (offered: 165 kJ ind⁻¹
606 day⁻¹) and CCR (offered: 95 =kJ ind⁻¹ day⁻¹; indicated by horizontal dotted lines); c) Torpor
607 incidence in CCR and control individuals (bars) and weekly minimum ambient temperature
608 (line).

609

610 Fig 3. Daily energy intake estimated from food consumption in function of air temperature
611 during the experimental period.

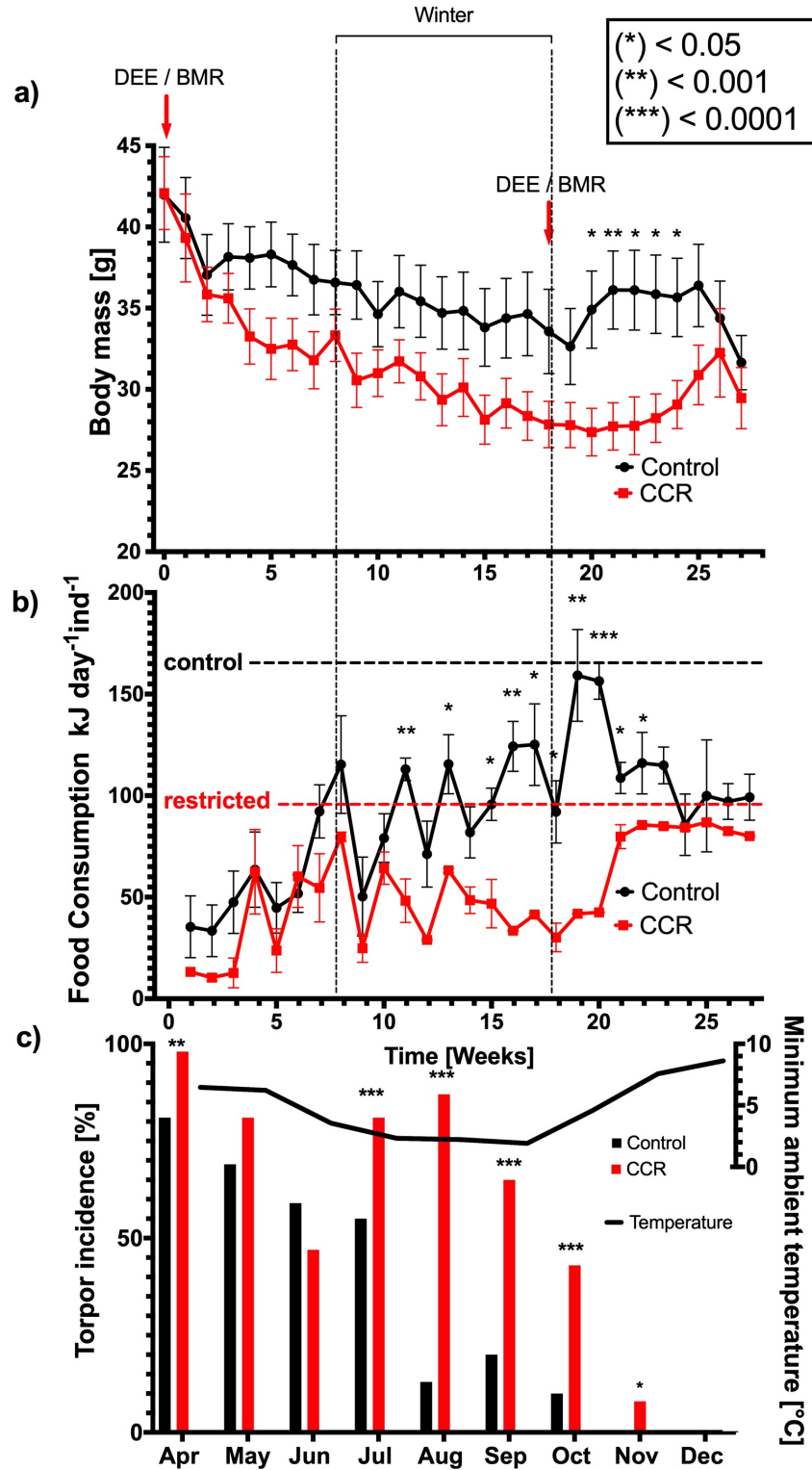
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613 Fig 4. a) Daily energy expenditure (DEE) in summer and winter *D. gliroides* under the CCR
614 and control conditions; b) basal metabolic rate; c) DEE aerobic scope; d) body masses, e)
615 scaling of summer animals for both CCR and control groups pooled; d) scaling of winter
616 animals. Significance ($P<0.05$) is denoted after a repeated measures ANOVA.



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Fig 1. Nespolo et al.

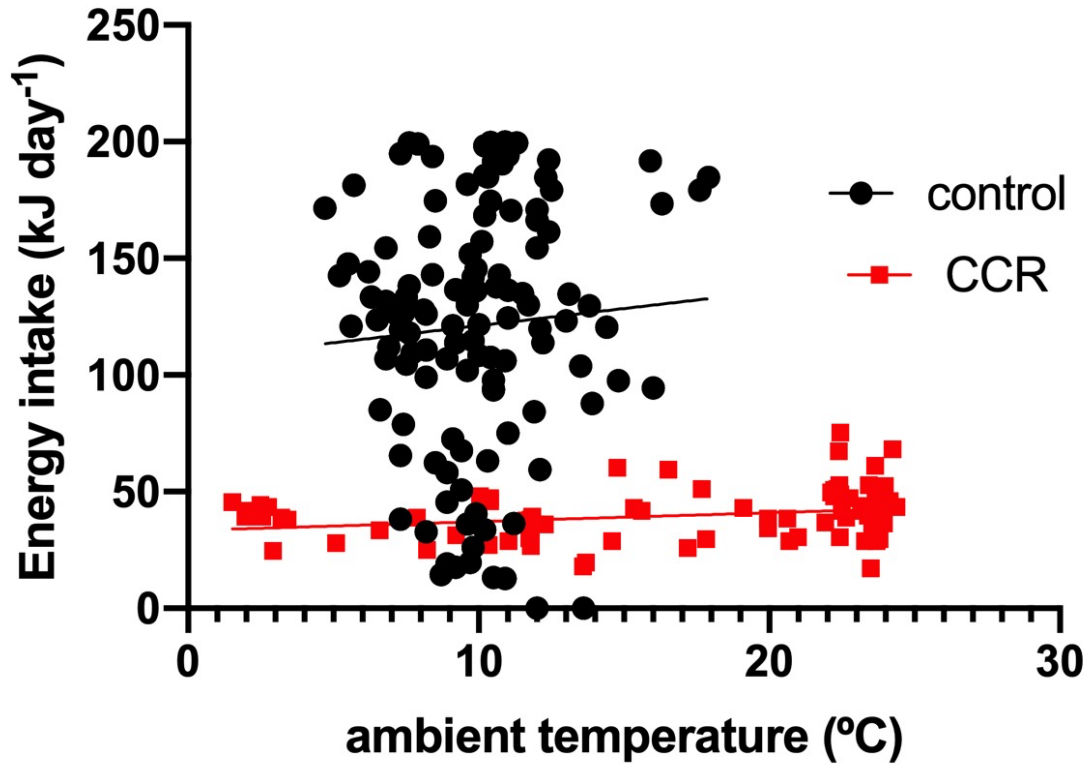


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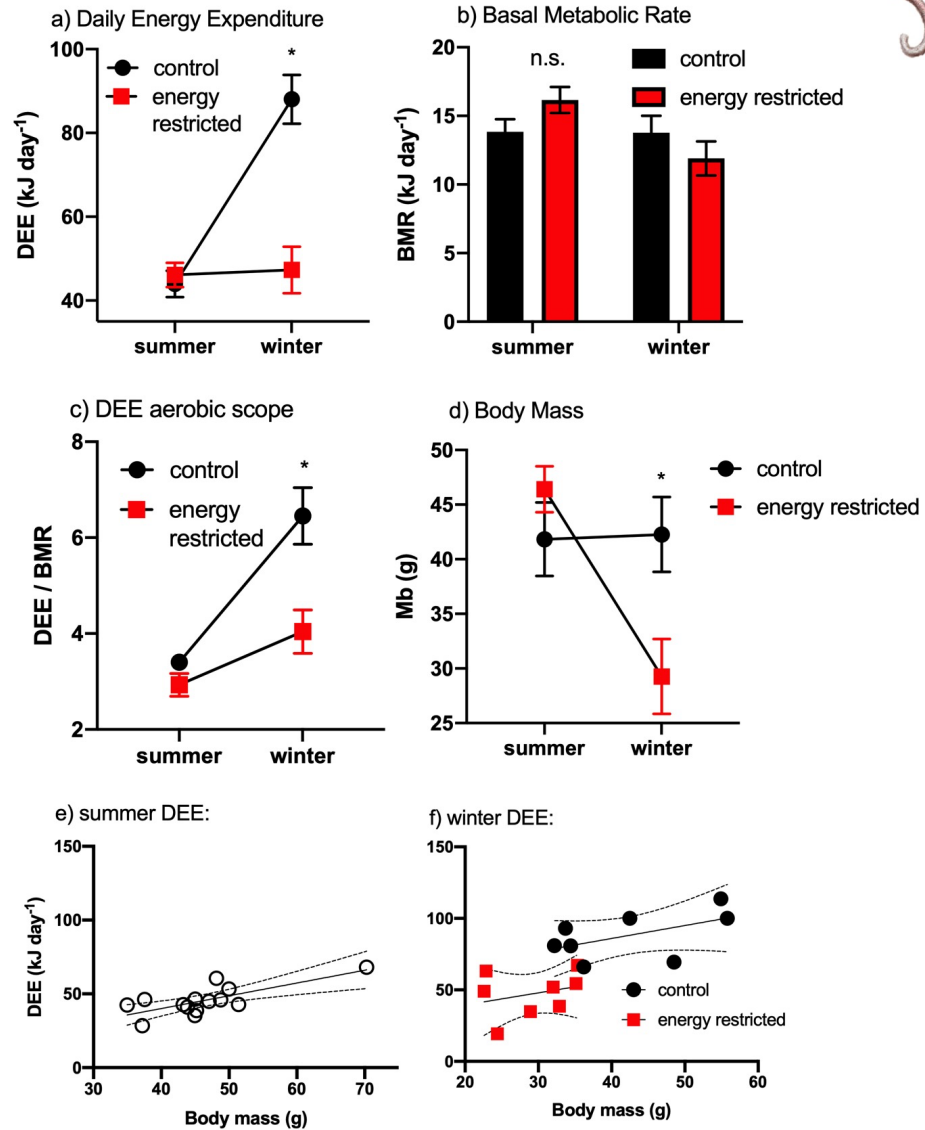
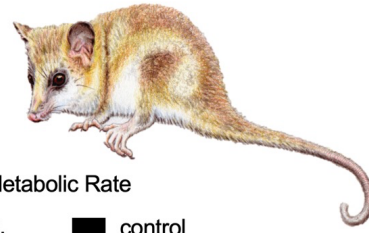
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Fig 2. Nespolo et al.



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Fig 3. Nespolo et al.



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Fig 4. Nespolo et al.

631 *Supplementary Material*

632

633 **A mesocosm experiment in ecological physiology: adaptive modulation of energy**
634 **budget in a hibernating marsupial under chronic caloric restriction**

635

636 Roberto F. Nespolo, Francisco E. Fontúrbel, Carlos Mejias, Rodrigo Contreras, Paulina
637 Gutierrez, José Ruiz, Esteban Oda, Pablo Sabat, Catherine Hambly, John R. Speakman &
638 Francisco Bozinovic

639

640 *Supplementary Material and Methods*

641

642 *Enclosures*

643 Each enclosure had a internal volume of 2 m³, and was manufactured in zinc by a large 1.8-
644 diameter cylinder buried 10 cm in the ground, which gave a 0.8 m height above ground.
645 Each ceiling was framed in timber, and had a mesh that allowed the entrance of light and
646 humidity, but avoided the escape of the animals or predator's attack. Then we included a
647 tri-dimensional arrangement of *Nothofagus* twigs and logs, native bamboo (*Chusquea*
648 *quilla*) in each enclosure, and the floor was covered by mosses and bamboo leaves, which
649 are known to be essential for *D. gliroides* nests building (Hershkovitz 1999, Honorato et al.
650 2016), resembling forest conditions (see Fig 1b-d in main text). We also included one
651 removable hibernaculum per enclosure, which consisted in a hollowed log of about
652 30x10x15 cm, cut longitudinally that was put over the ground in a way that allowed
653 animals to enter, cluster, rest, or hibernate. Each hibernaculum was sealed at each end by a
654 timber cover with a small hole in the middle, to allow animal entrance. In each enclosure,
655 we also put one max/min thermometer, one temperature data logger (HOBO®) for
656 continuous T°C recording and water ad libitum.

657

658 *Diet preparations*

659 *D. gliroides* is an omnivorous marsupial with well-known dietary preferences (Cortes et al.
660 2011, Rodriguez-Cabal and Branch 2011, Contreras et al. 2014), thus we offered three
661 dietary items to them in separate plates: apple compote, canned tuna (in water) and blend
662 (i.e., equal parts mix between berry jam and baby cereals plus 50% of water) (Contreras et
663 al. 2014))(see Fig 1e in main text). We also added a polyvitamin mixture in the diets (0.3
664 mg kg⁻¹ invade®). The apple compote and the tuna were offered as they are obtained from
665 the commercial suppliers. We always used the same commercial suppliers. Three samples
666 of each diet were dried and calorimetrically analyzed in a Parr calorimeter (Illinois, USA),
667 showing similar energy contents (dry weight)(tuna: 23.04 ± 3.4 kJg⁻¹; blend: 17.90 ± 0.12
668 kJg⁻¹; apple compote: 15.89 ± 0.48 kJg⁻¹)(see details in Table S1). We calculated food
669 consumption using marsupial allometric equations (Nagy 2001) and considering a
670 maximum energy expenditure that is six times basal metabolic rate (Bozinovic et al. 2004,
671 Nespolo et al. 2010, Franco et al. 2012).

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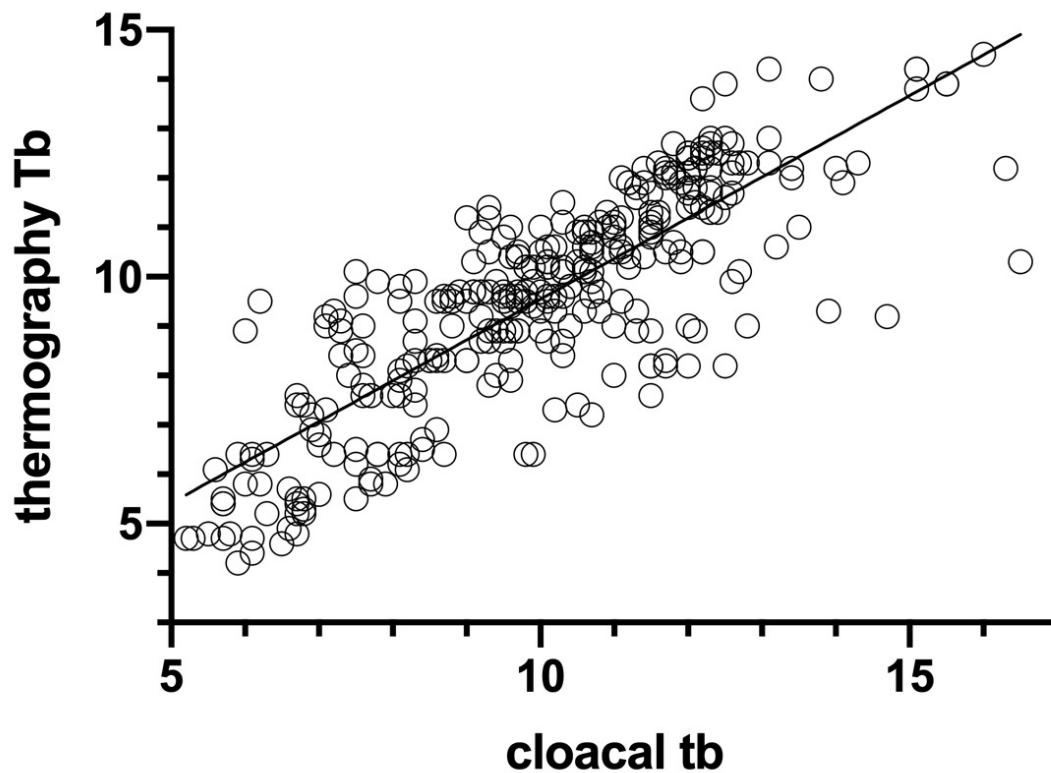
673 *Table S1. Nutrient content of the experimental diets provided to the enclosures. Each*
674 *enclosure received three dietary items: (1) a homogenized blend of jam and cereal diluted*
675 *in 50% water, (2) a weighed amount of tuna and (3) a weighed amount of apple compote*
676 *from a commercial supply (see methods for details).*
677

Commercial label	Jam	Cereal	Canned tuna	Apple compote
Calories (KJ/100g)	887	1,564.8	280.3	281.2
Protein (%)	0.3	9	15	0.3
Total fat (%)	0.2	1.8	0.4	0.3
Total Carbohydrate (%)	52.2	80.5	0.5	16
Total sugars (%)	51.7	26.0	0.5	16
Sodium (mg/100g)	13	80	314	4

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679

680 *Thermographic imaging*

681 For characterizing thermoregulatory abilities of hibernating *D. gliroides*, we visited the
682 enclosures every week, uncovered each hibernaculum, took a digital photo and an infra-red
683 photograph of clustered torpid individuals using a thermograph (FLIR systems, Oregon,
684 USA) set for an emissivity of 0.98 (Fig. 1f-g, total images: 328). This infrared imaging
685 permitted us to measure in situ external body temperatures (T_{TORPID}), by averaging the
686 temperature of a polygon drawn of the image of each animal using the FLIR tools software.
687 We also measured the mean temperature of the substrate 10 cm apart of the cluster
688 (T_{SUBSTR}). With this information, we calculated the thermal differential ($T_{DIFF} = T_{TORPID} -$
689 T_{SUBSTR}) for each animal, which is a measure of heat conservation in torpor. After recording
690 these images, we measured cloacal temperature on each animal, using a Cole-Parmer
691 copper-constantan thermocouple inserted 1 cm in the cloaca. This record was obtained
692 within a few minutes after taking the images (otherwise it was discarded). Cloacal
693 temperature was correlated with T_{TORPID} ($R^2 = 0.68$; $P < 0.01$; Fig. S1, $n = 410$). Finally,
694 each torpid animal was weighed and released back in the hibernaculum. We also recorded
695 the size of the cluster and whether they were found within the hibernaculum. We also
696 classified each animal as torpid or active by visual inspection (see Fig 1h in main text).



697

698 *Fig S1. Bivariate relationship between surface skin temperature measured by*
699 *thermographic images and cloacal temperature, measured by a copper-constantan*
700 *thermocouple, in each animal.*

701

702 *Doubly labelled water*

703 This method has been previously validated by comparison to indirect calorimetry in a range
704 of small mammals (e.g. Speakman and Krol, 2005). A weighed amount of DLW was injected
705 intraperitoneally into each individual. A blood sample (100ul) was collected from the tail
706 vein into glass capillaries and flame sealed 1 and 48 hours later. Background samples were
707 collected from some individuals prior to dosing. Analysis of the isotopic enrichment of blood
708 was performed blind using a Liquid Isotope Water Analyser (Los Gatos Research, USA)
709 (Berman et al. 2012). Initially the blood encapsulated in the capillaries was vacuum distilled
710 (Nagy 1983), and the resulting distillate was used. Samples were run alongside three lab
711 standards for each isotope and International standards to correct delta values to ppm.
712 Equation 7.17 of Speakman (1997)(Speakman 1997) assuming a single-pool model was used
713 to calculate rates of CO₂ production as recommended for use in animals less than 1 kg in
714 body mass (Speakman 1997). There are several approaches for the treatment of evaporative
715 water loss in the calculation (Visser and Schekkerman 1999). We assumed evaporation of
716 25% of the water flux (equation 7.17: Speakman 1997) which minimizes error in a range of
717 conditions (Visser and Schekkerman 1999, Van Trigt et al. 2002). CO₂ production was
718 converted to DEE using the Weir equation (Weir 1990).

719

720 *Basal metabolic rate*

721 Briefly, metabolic rate was recorded using a LiCor 6251 CO₂ analyzer in a 1L metabolic
722 chamber and a flow rate of 1,000 ml min⁻¹, after scrubbing water and CO₂ from the incoming
723 air. The metabolic chamber was located in an incubator, and ambient temperature was set to
724 thermoneutrality (30°C) which was continuously recorded by a thermocouple located inside
725 the incubator. These measurements were completed after a day of acclimation to the
726 laboratory and after food had been removed for 8 hrs. Metabolic trials all took place during
727 the typical rest phase of the animals (between 8am and 7pm). Each measurement had a
728 duration of three hours and most animals slept after the first hour in the chamber, which was
729 checked by visual inspection through a small window in the incubator. BMR (mlCO₂ h⁻¹) was
730 calculated from the three lowest steady-state values during the last 30 min of recording, and
731 converted to kJ assuming an RQ=0.71 (Walsberg and Wolf 1995).

732

733 *Statistical analyses*

734 We fitted Mixed-Effects Generalized Linear Models (GLMM) with a gaussian error
735 distribution and an 'identity' link function on the previously defined variables. We included
736 individual ID, enclosure, and sampling week as random effects to account for inter-
737 individual and inter-enclosure variability, along with the repeated measures in time (Zuur et
738 al. 2009). To estimate the best explanatory variables for torpor occurrence, we fitted a
739 GLMM with a binomial error distribution and a 'logit' link function (Beckerman et al.
740 2017), including treatment, body mass, and group size as predictors (fixed effects) and
741 individual ID, enclosure and sampling week as random effects, as described above. To
742 explore the factors that influence heat conservation in torpid animals, we fitted additional
743 models using the same parameters on a subset of data of torpid animals. Then, we fitted one
744 more GLMM to assess the factors determining T_{DIFF} , using CCR treatment, body mass, and
745 group size as predictors (fixed effects) and individual ID, enclosure and sampling week as
746 random effects, as previously described. We estimated GLMM parameters and their
747 significance using a restricted maximum likelihood approach with a Kenward-Roger
748 approximation to estimate degrees of freedom (Halekoh and Hojsgaard 2014). We

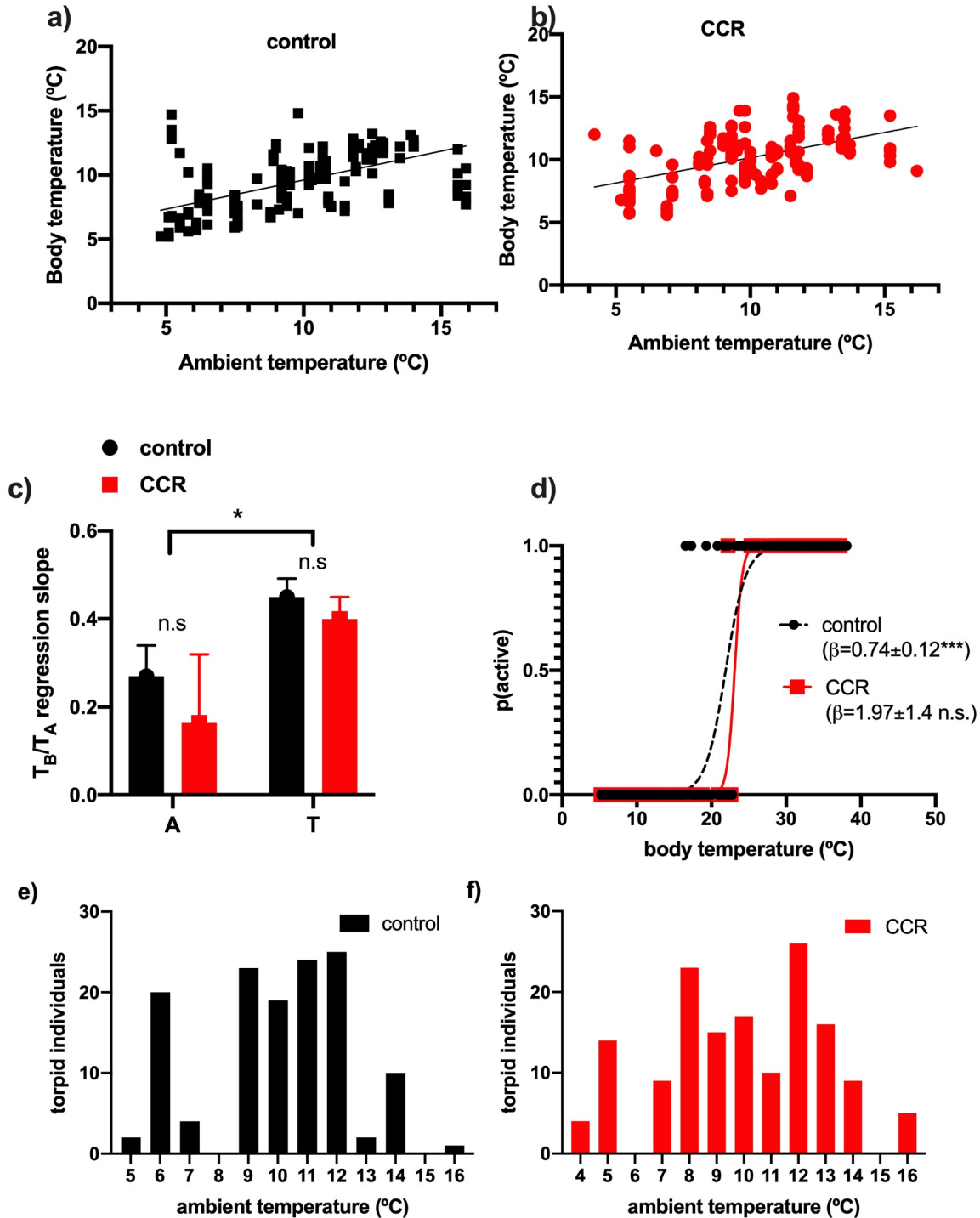
749 performed all analyses using R 3.6.0 (Team 2019), with the packages mgcv (Wood 2011),
750 lme4 (Bates et al. 2013), lmerTest (Kuznetsova et al. 2015), pbkrtest (Halekoh and
751 Hojsgaard 2014), and ggplot2 (Wickham 2016).

752

753 **Supplementary Results**

754 *Thermoregulation during torpor*

755 As soon as ambient temperature fell below $\sim 12^{\circ}\text{C}$, we observed packed clusters of torpid
756 animals, sometimes within a compact nest of interwoven leaves of native bamboo
757 (*Chusquea quila*) and mosses, or sometimes just buried in the ground. However,
758 thermoregulatory adjustments during hibernation between CCR and control animals were
759 not different, as revealed by thermographic images (summarized in Fig. S2 and Table S2,
760 $n=328$), and by the frequency of clustering or hibernacula use (summarized in Fig. S3,
761 $n=530$ and 618 , respectively). Although the GLMM model using torpor occurrence as a
762 binomial variable showed several significant effects of the CCR treatment, indicating
763 complex interaction among food deprivation, cluster size and body mass (Table S2), there
764 were non-significant effects of these variables on the thermal differential between animals
765 and substrate, estimated by the analysis of T_{DIFF} (Table S3). Thermoregulatory variables
766 such as the T_B/T_A slope comparison between control and CCR (Fig. S2a-c) and the
767 comparison of slopes of the logistic regression of torpid and active animals (Fig. S2d;
768 $n=795$ and 342 , control and CCR respectively) were non-significant. Also, the most
769 frequent substrate temperature for torpor in control individuals (median= 10.05 , min= 4.8 ,
770 max= 16.2°C , $n=130$) was nearly identical with CCR individuals (median= 10.1 , min= 4.2 ,
771 max= 15.9°C , $n=148$, Fig. S2e-f, non-significant differences after a median test). Behavioral
772 strategies for heat conservation such as clustering (control animals formed small groups
773 during torpor, whereas CCR animals did not show any trend, Fig. S3a-b), and hibernacula
774 use (control animals were preferably found within hibernacula, both active and torpid, Fig.
775 S3c-d) indicated absence of behavioral strategies for heat conservation in CCR. In other
776 words, ad libitum fed animals preferred hibernacula irrespectively of being active or torpid.
777

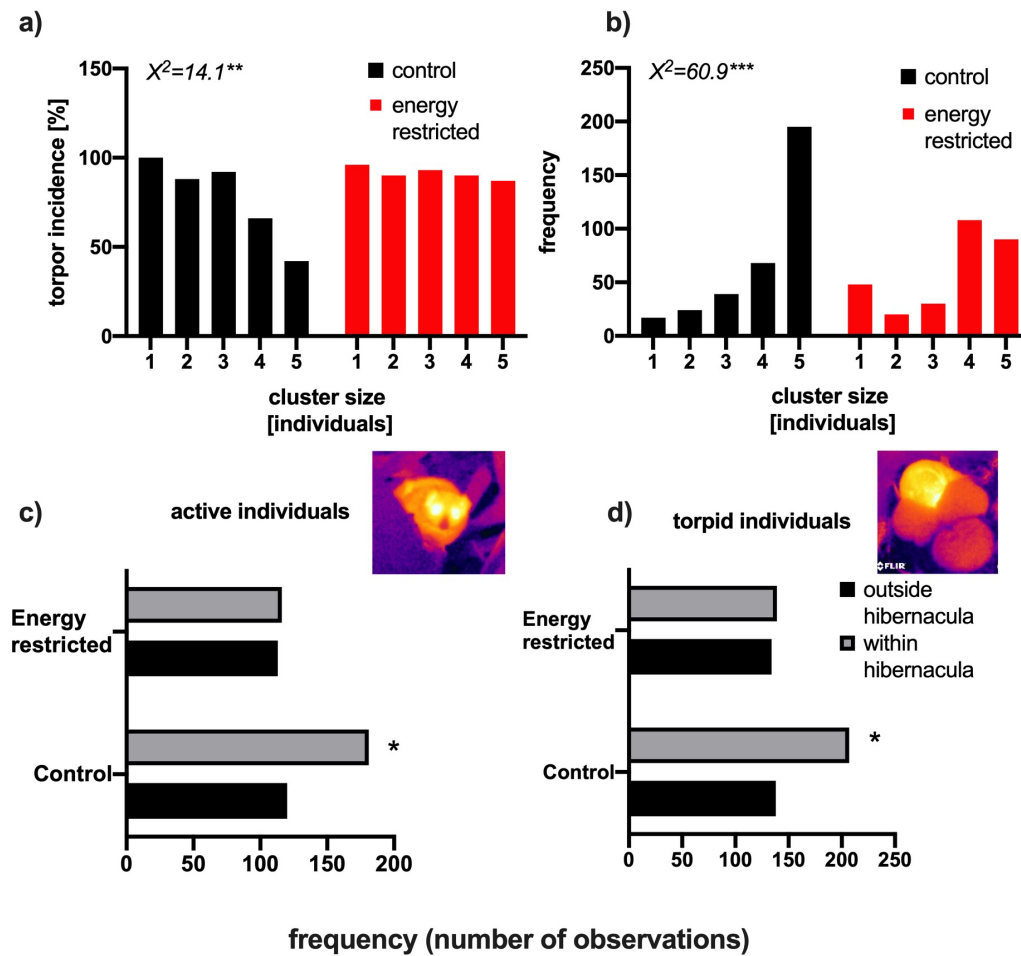


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780 *Fig S2. Thermal physiology of D. gliroides under control and energy restricted conditions.*
 781 *Linear regressions (a: control; b: treatment) between ambient temperature and body*
 782 *temperature measured weekly as cloacal temperature in a semi-natural experiment of*
 783 *chronic caloric restriction. Figure S2c shows a comparison of the T_A/T_B slopes (A: active;*
 784 *B: torpid) calculated above, showing significant differences only for torpid and active*
 785 *individuals: comparisons either within control ($F_{1,263}=19.9$; $P=0.018$; ANCOVA*

786 homogeneity of slopes model) or within energy restricted animals ($F_{1,387}=19.7$; $P=0.018$;
 787 ANCOVA homogeneity of slopes model). Non-significant differences were found for
 788 control/treatment comparisons within torpid or within active animals (indicated). Figure
 789 S2d) logistic regression between body temperature and probability of being active, showing
 790 a rewarming threshold in T_B of about 22°C , but it was non-significant for energy restricted
 791 animals. Figure S2e and f) shows substrate preferred temperatures in control and energy
 792 restricted individuals. Both distributions have identical medians ($=10.1^\circ\text{C}$).
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797 Fig S3. Frequency distributions of animals forming groups or using hibernacula during the
 798 CCR experiment. a) torpor incidence in function of cluster size; b) total frequency of cluster
 799 size; c) hibernacula use in active animals and d) hibernacula use in torpid animals.
 800 Significant values indicating different frequencies across categories, are indicated after a
 801 chi-square contingency table (indicated in the figure) and Fisher exact test ($*P<0.001$).

802 *Table S2. Results of a generalized linear mixed model fit by restricted maximum likelihood,*
 803 *for the binomial response variable “status” (active/torpid) using the logit link (n= 795). The*
 804 *model was: status ~ treatment (restricted/control) + body mass (M_B) + cluster size (1-5*
 805 *individuals) + enclosure (random factor) + ID (random factor) + week (random factor). The*
 806 *model included all possible interactions.*
 807

Variable	Estimate	SE	z-value	P-value
(Intercept)	10.220	4.169	2.451	0.014
caloric restriction treatment	-21.469	6.640	-3.234	0.001
mass	-0.485	0.134	-3.621	<0.001
group.size	-1.889	0.842	-2.243	0.025
dietTreatment:mass	0.652	0.202	3.225	0.001
dietTreatment:group.size	3.092	1.468	2.106	0.035
mass:group.size	0.091	0.027	3.301	0.001
dietTreatment:mass:group.size	-0.114	0.046	-2.496	0.013

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Table S3. Results of a generalized linear mixed model fit by restricted maximum likelihood,
for the response variable “ T_{DIFF} ” (thermal differential), obtained using thermographic
pictures in clustered hibernating animals (n= 328). The model was: T_{DIFF} ~ treatment
(restricted/control) + body mass (M_B) + cluster size (1-5 individuals) + enclosure (random
factor) + ID (random factor) + week (random factor). The model included all possible
interactions.

Variable	Estimate	SE	df	t-value	Pr(> t)
(Intercept)	5.840e-01	1.898e-01	8.272e+01	3.077	0.00283**
dietTreatment	-1.676e-02	6.980e-02	6.183e+00	-0.240	0.81804
mass	-2.189e-04	4.004e-03	6.031e+01	-0.055	0.95658
group.size	2.508e-02	2.456e-02	2.688e+02	1.021	0.30809

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

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