

1 **Specific shifts in the endocannabinoid system in hibernating brown bears**

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23 **Keywords**

24 Hibernation, Brown bear, Metabolism, Lipidomic, Docosaehaenoic acid, Endocannabinoid
25 system, N-oleoylethanolamide, 2-arachidonoylglycerol, Anandamide, Cannabinoid receptor 1.

26

27 **Abstract**

28 In small hibernators, global downregulation of the endocannabinoid system (ECS), which is
29 involved in modulating neuronal signaling, feeding behavior, energy metabolism, and circannual
30 rhythms, has been reported to possibly drive physiological adaptation to the hibernating state.
31 We hypothesized that specific changes should occur in hibernating brown bears (*Ursus arctos*)
32 due to specific features, including hibernation during half the year at only mild hypothermia while
33 remaining physically inactive without drinking or eating, and the absence of arousal episodes
34 although bears remain sensitive to external disturbances. We explored circulating lipids and the
35 ECS in plasma and metabolically active tissues (muscle and adipose tissue), in free-ranging
36 subadult Scandinavian brown bears when both active and hibernating. In winter bear serum, in
37 addition to a 2-fold increase in total fatty acid concentration, we found significant changes in
38 relative proportions of circulating fatty acids, such as a 2-fold increase in docosahexaenoic acid
39 and a decrease in arachidonic acid. In adipose and muscle tissues of hibernating bears, we found
40 lower concentrations of both two major ligands for endocannabinoid receptors, 2-
41 arachidonoylglycerol (2-AG) and anandamide (AEA). Gene expression was reduced for enzymes
42 that synthesize endocannabinoid compounds, whereas an increase was observed for catabolic
43 enzymes. Reduction in ECS tone may promote mobilization of fat stores and favor carbohydrate
44 metabolism in skeletal muscle of hibernating bears. Additionally, high circulating of the
45 endocannabinoid-like compound N-oleoylethanolamide (OEA) in winter could favor lipolysis and
46 fatty acid oxidation in peripheral tissues. We also speculated on a role of OEA in the maintenance
47 of torpor (reduction in locomotion), while promoting the capacity of bears to sense stimuli from
48 the environment.

49 **Background**

50 To deal with seasonal cold and food shortage during winter, hibernating mammals show a
51 combination of behavioral and physiological changes. To save energy during hibernation,
52 hibernating animals use periods of torpor characterized by decreased metabolic rate and body
53 temperature, reduction in respiratory and heart rates, and physical inactivity [1,2]. Brown bears
54 (*Ursus arctos*) exhibit unique features, as they hibernate at mild hypothermia (32-35°C) and stay
55 inside their dens for up to 7 months, without drinking, eating, defecating or urinating, and with
56 no arousal episodes [3–6]. While denning, they reduce their metabolic rate by about 75% [7],
57 and rely on mobilization of fat stores, which is reflected in increased circulating fatty acid
58 concentration during winter [8–10].

59 Beyond energy substrates, lipids also have pleiotropic actions in the regulation of metabolism,
60 and changes in membrane fatty acid composition have already been described in hibernating
61 animals [11–13]. Membrane lipids can also provide long-chain fatty acids for the synthesis of
62 bioactive lipid mediators, such as endocannabinoids [14–17]. The endocannabinoid system (ECS)
63 was originally described as being composed of G-protein coupled receptors, CNR1 and CNR2, and
64 their endogenous ligands, of which the main ones are derived from arachidonic acid (AA) and
65 called 2-arachidonoyl glycerol (2-AG) and anandamide (AEA) [14,15,17]. 2-AG and AEA belong to
66 the large family of 2-acylglycerols (2-AcGs) and N-acylethanolamines (NAEs), respectively. N-acyl-
67 phosphatidylethanolamine-hydrolyzing phospholipase D (NAPEPLD) and sn-1-specific
68 diacylglycerol lipase- α and β (DAGLA and DAGLB) are the main enzymes involved in the
69 biosynthesis of NAEs and 2-AcGs, respectively, fatty acid amide hydrolase (FAAH) is responsible
70 for NAEs and 2-AcGs catabolism, and monoacylglycerol lipase (MGLL) specifically catabolizes 2-

71 AcGs [15,19]. However, the ECS is more complex and structurally related compounds like N-
72 oleoylethanolamine (OEA), have been identified and called «endocannabinoids-like compounds»
73 (eCBs-like). The latter are metabolized by the same biosynthetic and catabolic enzymes as eCBs
74 [17]. Although eCBs-like compounds are not able to bind to CNR1 and CNR2, they can bind to
75 other G-protein coupled receptors (e.g. GPR119 and GPR55) or nuclear receptors, like
76 peroxisome proliferator-activated receptor α (PPARA) [17].

77 Endogenous cannabinoids are involved in the regulation of many physiological processes,
78 including thermogenesis, energy storage, metabolism, sleep cycles, circannual rhythms, stress
79 response, feeding behavior, and skeletal muscle differentiation [20–23]. At the central level (e.g.
80 hypothalamus), CNR1 is able to promote food intake and reduce energy expenditure [23,24]. In
81 addition, CNR1 activation in adipose tissue leads to fatty acid and glucose uptake, and to
82 upregulation of lipogenesis [23]. In liver, CNR1 signaling leads to increased expression of genes
83 involved in the synthesis of fatty acids [25], and in skeletal muscle tissue, CNR1 activation triggers
84 a decrease in glucose uptake and insulin sensitivity [23]. By contrast, OEA promotes lipolysis, fatty
85 acid oxidation in skeletal muscle and liver, and it triggers an anorexigenic signal, notably through
86 the nuclear receptor PPAR- α [26,27]. Considering the pleiotropic roles of ECS in neuronal
87 signaling, regulation of behavior, appetite, energy metabolism, and circannual rhythms, it is not
88 surprising to see important changes during hibernation. A decrease in ECS tone has notably been
89 observed in hibernating marmots (*Marmota monax* and *flaviventris*) and ground squirrels
90 (*Spermophilus richardsonii*) [21,28,29]. We hypothesized that a similar decrease could occur in
91 hibernating bears, although specific changes due to their unique features during hibernation
92 (mild hypothermia, no food intake, no periodic arousal, and maintenance of alertness) could also

93 be expected. Therefore, we explored seasonal variations in fatty acid composition and ECS tone,
94 in both the circulating compartment and in muscle and adipose tissues, in winter hibernating and
95 summer-active brown bears.

96 **Results**

97 **Seasonal differences in serum lipids**

98 To explore the fatty acid (FA) composition of winter-hibernating (WBS) and summer-active (SBS)
99 bear serum, six paired mixes (a winter and a summer one for each of the six considered years)
100 were prepared (See supplementary Table S1). From the twelve samples analysis, we then
101 compared both the concentrations and proportions of fatty acids (see supplementary Table S2
102 and S3 for detailed lipidomic results). As shown in Figure 1A, the total concentration of FAs was
103 about twofold higher in WBS relative to SBS (28.82 ± 1.71 vs. 15.99 ± 1.09 mmol/L). The
104 concentration of each lipid group, saturated fatty acids (SFAs), monounsaturated fatty acids
105 (MUFAs), and polyunsaturated fatty acids (PUFAs), including n-3 and n-6 species, was higher in
106 hibernating bears (see supplementary Table S2). Only concentrations of alpha-linolenic acid (ALA)
107 and eicosapentaenoic acid (EPA) were lower in WBS, by about 0.49- and 0.26-fold, respectively.
108 Meanwhile, both molar percent of total n-6 and n-3 species were found to be lower in WBS
109 compared to SBS (Figure 1B). Among SFAs, palmitic acid (PA) occurred in higher proportion,
110 whereas stearic acid (SA) was in lower proportion in winter. Similar proportions of oleic acid (OA),
111 belonging to the n-9 MUFAs, were found in winter and summer bear serum (Figure 1C).
112 Concerning n-6 PUFAs, the proportion of arachidonic acid (AA) was lower during winter, whereas
113 proportion of linoleic acid (LA) remained unchanged. Individual species of the n-3 family,
114 docosapentaenoic acid (DPA, 1.5-fold) and docosahexaenoic acid (DHA, 2.2-fold) were found in
115 higher proportions, whereas the level of their precursor alpha-linolenic acid (ALA, 0.27-fold) was
116 lower during winter (Figure 1C and supplementary Table S3). The WBS proportion of EPA was

117 also much lower (0.15-fold). From molar percent values, the DHA/AA ratio showed opposite
118 changes between seasons, being 3.2-fold higher in winter (Figure 1D).

119

120 **Changes in plasma endocannabinoids and endocannabinoids-like compounds**

121 We next assessed circulating endocannabinoids (eCBs) and endocannabinoids-like compounds
122 (eCBs-like) in bear plasma. Paired samples were collected in winter and in summer from eight
123 bears (Supplementary Table S1) and quantification of anandamide (AEA), 2-arachidonoyl glycerol
124 (2-AG) and N-oleoylethanolamine OEA are presented in Figure 2. Lower concentrations were
125 observed for AEA in winter compared to summer, whereas the reverse was observed for OEA
126 (3.3-fold). No difference was found for 2-AG plasma concentration.

127

128 **Changes in endocannabinoid concentrations in muscle and adipose tissues**

129 Quantification of endocannabinoids was then performed in bear muscle and adipose tissues
130 Paired tissues samples were collected from bears in winter and in summer (Supplementary Table
131 S1) and quantification of AEA, 2-AG and OEA are presented in Figure 3. AEA was found to be
132 depleted in both muscle and adipose tissues during winter versus summer, close to the statistical
133 threshold ($p=0.064$ and $p=0.069$, respectively). 2-AG concentration was significantly lower in
134 muscle and adipose tissues samples during winter, by about 1.6- and 9-fold, respectively. By
135 contrast, no seasonal changes were found in OEA concentrations in both muscle and adipose
136 tissues.

137

138 **Changes in endocannabinoid pathway-related gene expressions in muscle tissue**

139 To explore muscle tissue metabolism of endocannabinoids, we quantified gene expression from
140 total RNA extracted from summer and winter paired muscle tissue of eight bears (Supplementary
141 Table S1). For genes that encode the eCBs membrane receptors, as shown in Figure 4 the mRNA
142 level of CNR1, but not of CNR2, was decreased in muscle during winter compared to summer. For
143 genes encoding the enzymes that catabolize AEA and 2-AG, mRNA level of fatty acid amide
144 hydrolase (FAAH) was induced by more than twofold during the winter period, but
145 monoacylglycerol lipase (MGLL) gene expression did not change.

146 For genes encoding enzymes of the biosynthetic pathway, expression of diacylglycerol lipase
147 alpha (DAGLA) was strongly reduced in muscle tissue during winter (0.40 fold), whereas the beta
148 isoform (DAGLB) was overexpressed (1.53 fold). Finally, gene expression of N-
149 acylphosphatidylethanolamine phospholipase D (NAPEPLD) did not change.

150 **Discussion**

151 Thanks to repeated capture sessions for several years in Dalarna and Gävleborg counties,
152 Sweden, we were able to gather paired samples of serum, plasma and tissues from a high number
153 of free-living brown bears (*Ursus arctos*). For all the 28 bears included in this study, samples were
154 collected both in February during winter hibernation and in June during the summer active
155 period, and all analysis were performed on paired mixes or individual samples (Supplementary
156 Table 1). We examined circulating lipid and ECS compounds in both summer-active and winter-
157 hibernating brown bears to explore the extent to which regulation of the ECS reflects bear
158 hibernation peculiarities, including survival due to lipid oxidation, maintenance of muscle
159 glycolysis, and maintained alertness during dormancy. The seasonal shift we highlighted in serum
160 FAs composition, together with a decrease in tissue AEA and 2-AG, and a three-fold increase in
161 circulating OEA during winter, no doubt contribute to the behavioral and metabolic changes that
162 occur in hibernating bears.

163 Many hibernators experience extended periods of food shortage during hibernation and rely on
164 mobilization of fat stores from white adipose tissue (WAT) [1]. Accordingly, we found that the
165 concentration of total fatty acids was elevated in the serum of hibernating bears, a finding in line
166 with previous studies [5,30]. Considering both the amount and relative proportions of circulating
167 lipids, our results are consistent with changes in serum lipid profiles during hibernation that have
168 been previously published [5,9,10], notably an enrichment in DHA (22:6n-3) and depletions in
169 ALA (18:3n-3) and EPA (20:5n-3), during winter compared to summer. Concomitantly, we
170 observed a drop in arachidonic acid (AA) proportion, thus leading to a sharp increase in the
171 DHA/AA ratio. The health benefits that have been attributed to n-3 PUFAs (e.g. DHA) could

172 potentially be transposed in the context of hibernation. Indeed, it has already been hypothesized
173 that DHA could be involved in the bear's resistance to muscle atrophy during hibernation [10].
174 DHA supplementation has also been reported to prevent muscle atrophy in fasting mice [31], in
175 part by increasing muscle glycogen stores. Strikingly, hibernating bears have more than a 3-fold
176 higher glycogen muscle content compared to summer-active animals [10]. In addition to its anti-
177 inflammatory effects, DHA is also known to exert a positive effect on protein balance by
178 decreasing expression of factors involved in protein breakdown [32] and enhancing protein
179 synthesis, notably by promoting mammalian Target Of Rapamycin (mTOR) activation [33]. It is
180 noteworthy to mention that, in response to DHA supplementation, an enrichment of this fatty
181 acid in phospholipids of cell membranes occurs in parallel with a decrease in AA content
182 [34,27,35,36]. By remodeling the amount of AA-containing phospholipids, DHA is able to reduce
183 the synthesis of the two main arachidonate-based eCBs : AEA and 2-AG [35,36].

184 The eCB-like OEA is generally synthesized in response to dietary fat intake (oleic acid) by
185 enterocytes of the small-intestine [35,36]. In addition, n-6 PUFAs-enriched diets have been
186 shown to increase the level of 2-AG or AEA in the brain, plasma, and peripheral tissues in
187 nonhibernating animal models [35,37–39]. However, no dietary intake of FAs could directly
188 modulate the level of fatty-acid derived compounds, such as endocannabinoids compounds,
189 during bear hibernation. Data on eCBs compounds from experimental fasting in nonhibernating
190 mammals are very divergent, depending on the tissue considered (e.g. brain or peripheral
191 tissues) and the duration of food deprivation, but tissue levels of eCBs are mainly regulated by
192 the availability of their membrane phospholipid precursors and by the activity of biosynthetic
193 and catabolic enzymes [35,40–42].

194 We hypothesized that: drastic reduction in metabolic activity, lack of intake of dietary PUFAs,
195 significant increase in the serum DHA/AA ratio, and perhaps reduction in tissue AA-phospholipids
196 concentration, could lead to a global reduction in ECS tone during the hibernation period. The
197 reduction in ECS tone has already been documented in hibernating marmots [21,29], but was not
198 confirmed in large-bodied hibernators. In addition to the decrease in tissue concentration of 2-
199 AG and AEA that we reported, we observed an unexpected 3-fold increase in OEA circulating
200 levels in hibernating bears, whereas no seasonal differences in OEA content in muscle or adipose
201 tissues were observed. It has already been shown in rodents that food deprivation inhibits OEA
202 synthesis in the small intestine, but stimulates its synthesis in liver [27,34,43,44]. Therefore,
203 during bear hibernation, circulating OEA could originate from tissue synthesis (probably hepatic)
204 and release into the blood flow. In other fat storing hibernators like marmots, low levels of
205 circulating OEA during hibernation may indicate a different regulation and it is not known if and
206 how OEA concentrations vary in food storing hibernators.

207 Taken together, our data allowed us to make hypotheses about possible mechanisms by which
208 ECS could contribute to the metabolic and behavioral changes that occur in bears during
209 hibernation. First, as endogenous agonists for CNR1, AEA and 2-AG favor food intake and
210 stimulate lipogenesis [23], CNR1 signaling is expected to be upregulated in hibernating mammals
211 during the active summer period, in order to promote energy storage, and downregulated during
212 winter, to stimulate lipolysis and FAs oxidation. Accordingly, we found lower circulating AEA and
213 lower amounts of 2-AG and AEA in hibernating bear muscle and adipose tissues. The tissue
214 concentration drops in 2-AG and AEA observed during winter could be due to a decrease in tissue
215 AA-phospholipids concentration, as we hypothesized above. The degradation of AEA and 2-AG

216 could also be increased during hibernation, as reflected in the higher mRNA levels of FAAH in
217 muscle (that degrades AEA and to a lesser extent 2-AG) [19]. With the additional reduction of
218 CNR1 mRNA levels in muscle during hibernation, the data strongly support reduced CNR1
219 signaling in muscle and adipose tissue. Reduced CNR1 signaling in adipose tissue could limit
220 lipogenesis and promote lipolysis, thus providing fatty acids as a fuel source for all tissues during
221 hibernation in bears, as also suggested for hibernating marmots [21].

222 In muscle tissue, CNR1 signaling is involved in the regulation of metabolism key actors, like AMP-
223 activated protein kinase (AMPK) and pyruvate dehydrogenase kinase 4 (PDK4) [45]. AMPK is
224 activated during nutrient deprivation and hypoxia and modulates cell metabolism, enhancing
225 both glucose transport and β -oxidation of fatty acids [46]. PDK4 is a major regulator of the
226 pyruvate dehydrogenase (PDH) that controls the flux of carbohydrates into the TCA cycle and
227 thus the switch between fatty acid and glucose oxidation [47]. In nonhibernating mammals, CNR1
228 activation leads to higher expression of the AMPK α 1 subunit and a decrease of PDK4 expression
229 that in turn enhances PDH activity [23,45,48,49]. Interestingly, recent studies have shown that
230 PDK4 is upregulated in hibernating bear skeletal muscle [10,50], whereas A1 and B subunits of
231 PDH are downregulated [10], and AMPK activity is reduced [51]. In hibernating bear muscle,
232 AMPK and PDK4 expressions appear thus to be disconnected from direct regulation by CNR1.

233 CNR1 receptor antagonism also leads to an increased uptake of glucose in muscle via PI3K
234 signaling [49] and glycolysis appears preserved in bear skeletal muscle during hibernation, as
235 suggested by an overall increase in the protein abundance of all glycolytic enzymes [10]. As
236 proposed by Chazarin et al. and Vella et al., bears still oxidize glucose and produce lactate in

237 skeletal muscle during hibernation [10,51]. In line with this hypothesis, the reduction of muscle
238 CNR1 signaling we report here could help maintain glucose uptake by muscle during hibernation.
239 OEA is a high-affinity agonist for peroxisome proliferator-activated receptor α (PPAR- α),
240 regulating food intake and stimulating fat catabolism [26,27,34,52,53]. Consequences of high
241 levels of circulating OEA have been studied in nonhibernating rodents. Intraperitoneal OEA
242 administration in rats notably impairs locomotor activity, which is supported by a decrease in
243 ambulation, an increase of the time spent in inactivity, and the presence of signs of catalepsy
244 [52,54]. We thus can hypothesize that a higher amount of plasma OEA during bear hibernation
245 can participate in the maintenance of prolonged physical inactivity. It has also been shown that
246 intracerebroventricular injections of OEA promote alertness, with the observation of enhanced
247 dopamine and c-Fos expressions in wake-related brain areas [55]. Bears are known to stay
248 sensitive to disturbance during hibernation [56–58]. High circulating amounts of OEA might thus
249 participate in alertness to external stimuli from the environment in hibernating bears. OEA during
250 winter possibly also favors body fat mobilization for energy needs, with stimulation of FA and
251 glycerol release from adipocytes [26,27]. Finally, a potential role for OEA in the promotion of
252 fasting-induced ketogenesis during hibernation could also be considered, as OEA has been
253 demonstrated to increase 3-hydroxybutyrate production in *in vivo* rodent models [26,27].

254 **Conclusions**

255 In conclusion, our results show a reduction in ECS tone in hibernating bears and suggest a
256 coordinated downregulation of CNR1 signaling in adipose and muscle tissues, which could favor
257 energy mobilization (lipolysis) and optimization of glucose uptake by skeletal muscles.
258 Conversely, an increased OEA level may participate in the behavioral and physiological

259 adaptations during bear hibernation state, like reduction in motor activity and torpor,
260 conservation of alertness at the level of central nervous system, and promotion of lipolysis and
261 fatty acid β -oxidation.

262

263 **Methods**

264 **Bear sample collection**

265 A total of 28 free ranging subadult brown bears (*Ursus arctos*) from Dalarna and Gävleborg
266 counties, Sweden, were included in this study, including 4 bears captured two consecutive years
267 (Supplementary Table S1). For all bears, samples were collected at two time points, in February
268 during winter hibernation and in June during summer active period. Blood, subcutaneous adipose
269 tissue, and muscle tissue (vastus lateralis) samples were collected from active (summer) and
270 hibernating (winter) brown bears was performed as described previously [10,59]. All samples and
271 data were collected under protocols approved by the Swedish Ethical Committee on Animal
272 Experiment (applications Dnr C3/2016 and Dnr C18/2015), the Swedish Environmental Protection
273 Agency (NV-00741-18), and the Swedish Board of Agriculture (Dnr 5.2.18-3060/17). All
274 procedures complied with Swedish laws and regulations. Blood samples were collected from the
275 jugular vein into dry tubes for serum (Vacuette® Z serum Sep Clot Activator, Greiner Bio-One
276 GmbH, Kremsmünster, Austria) or into EDTA-coated tubes (BD Vacutainer®, FisherScientific,
277 Illkirch, France) for plasma. Serum mixes were prepared as previously described [59]. All samples
278 were stored at -80°C until analysis were simultaneously performed on winter and summer paired
279 samples.

280 **Lipid extraction and analysis**

281 Lipids were extracted and analyzed as previously described [60]. After addition of an internal
282 standard (tri-17:0 triacylglycerol), total lipids were extracted twice from bear serum mixes with
283 ethanol/chloroform (1:2, v/v). The organic phases were dried under nitrogen and lipids were

284 transmethylated. Briefly, samples were treated with toluene-methanol (1:1, v/v) and boron
285 trifluoride in methanol (14%). Transmethylation was carried out at 100 °C for 90 min in screw-
286 capped tubes. Then 1.5 mL K₂CO₃ in 10% water was added and the resulting fatty acid methyl
287 esters were extracted by 2 mL of isooctane and analyzed by gas chromatography (GC) with a
288 HP6890 instrument equipped with a fused silica capillary BPX70 SGE column (60 x 0.25 mm). The
289 vector gas was hydrogen. Temperatures of the Ross injector and the flame ionization detector
290 were set to 230 °C and 250 °C, respectively. Data were expressed in mmol/L for total or individual
291 fatty acids (FAs) concentration or molar percentage of total lipids for individual FAs. Detailed
292 lipidomic results are presented in supplementary Table S2 (serum fatty acids concentrations) and
293 S3 (serum fatty acids relative proportions).

294 **Endocannabinoid quantification**

295 Standard endocannabinoids (eCBs), i.e.- PEA, PEA-d5, OEA, OEA-d4, AEA, AEA-d4, 2AG, and 2AG-
296 d5, were purchased from Cayman (Bertin BioReagent, Saint-Quentin en Yvelines, France). Mass
297 spectrometry quality grade solvents were purchased from Fischer Scientific (Illkirch, France).
298 Tissue samples (adipose and muscle tissues); c.a 100 mg) were crushed in an Omni Bead Ruptor
299 24 apparatus (Omni International, Kennesaw, USA) with circa twenty 1.4 mm OD zirconium oxide
300 beads (S=6.95 m/s, T=30s, C = 3; D = 10s) and 900 µl of methanol/Tris-buffer (50 mM, pH=8) 1/1
301 containing 20 ng of PEA-d5, 2 ng OEA-d4, 10 ng AEA-d4, and 20 ng 2AG-d5. Then, each
302 homogenate was added with 2 mL of CHCl₃/MeOH (1:1, v/v) and 500 µL of Tris (50 mM, pH=8),
303 vortexed and centrifuged 10 min at 3000g. The organic layer was recovered and the upper
304 aqueous phase was extracted twice with chloroform (1mL). Finally, organic phases were pooled
305 and evaporated under vacuum.

306 Plasma (500 μ L) were mixed with 500 μ L cold methanol containing 11 ng AEA. After protein
307 precipitation at -20°C for 2 hours, endocannabinoids were extracted with methanol/chloroform
308 (1:1, v/v) (5 ml) and saline (1.25 mL). The organic phase was recovered and the aqueous phase
309 was extracted twice with chloroform (3mL). Organic phases were finally pooled and evaporated
310 under vacuum.

311 Dried extracts were solubilized with methanol (200 μ L) and centrifuged for 5 min at 20,000 g.
312 Four microliters of the supernatant were injected into a 1200 LC system coupled to a 6460-QqQ
313 MS/MS system equipped with an ESI source (Agilent technologies). Separation was achieved on
314 Zorbax SB-C18 2.1x50 mm, 1.8 μ m column (Agilent technologies) at a flow rate of 0.4 mL/min,
315 40°C , with a linear gradient of (solvent A) water containing 0.1 % formic acid and (solvent B)
316 methanol containing 0,1% formic acid as follows: 10% of B for 1 min, up to 85% of B in 8 min, and
317 then 100% B for 4.5 min. Acquisition was performed in positive Selected Reaction Monitoring
318 (SRM) mode (source temperature: 350°C , nebulizer gas flow rate: 10 L/min, 40 psi, sheath gas
319 flow 10 L/min, sheath gas temperature 350°C , capillary 4000 V, nozzle 1000 V).

320 Transitions used were: 2AG-d5 384.3 \rightarrow 91.1 (frag 120V, CE 62V), 2AG 379.1 \rightarrow 91 (frag 120V, CE
321 62V), AEA-d4 352.2 \rightarrow 66.1 (frag 115V, CE 14V), AEA 348.2 \rightarrow 62 (frag 120V, CE 14V), OEA-d4
322 330.2 \rightarrow 66.1 (frag 120V, CE 14V), OEA 326.2 \rightarrow 62 (frag 115V, CE 14V), PEA-d5 305.2 \rightarrow 62 (frag
323 124V, CE 14V), and PEA 300.2 \rightarrow 62 (frag 124V, CE 14V).

324 eCBs from tissues were quantitated according to the isotope dilution method. Results are
325 expressed as pg per mg of wet weight of tissue. eCBs from plasma were quantitated using
326 calibration curves obtained with authentic standards extracted by the same method used for

327 plasma samples. Linear regression was applied for calculations. Results are expressed as ng of
328 endocannabinoid per mL of plasma.

329

330 **Quantification of mRNAs by real-time RT-PCR**

331 Muscle total RNA was isolated using the TRIzol reagent (Invitrogen, Courtaboeuf, France)
332 according to the manufacturer's instructions. First-strand cDNAs were synthesized from 1 µg of
333 total RNA using the PrimeScript RT kit (Ozyme, saint quentin en Yveline, France) with a mixture
334 of random hexamers and oligo(dT) primers, and treated with 60 units of RnaseH (Ozyme). Real-
335 time PCR assays were performed with Rotor-Gene 6000 (Qiagen, Courtaboeuf, France). The
336 primers and real-time PCR assay conditions are listed in supplementary Table S4. The results were
337 normalized by using TBP (TATA box binding protein) mRNA concentration, measured as reference
338 gene in each sample.

339 **Statistical analysis**

340 All data are presented as means ± SEM. To compare the serum, plasma, muscle, and adipose
341 tissues from summer and winter bears (SBS and WBS, SBP and WBP, SBM and WBM, and SBA
342 and WBA, respectively), statistical significance was determined using bilateral paired Student t-
343 tests, * indicating a p value < 0.05, **p < 0.01, and *** p < 0.001.

344

345 **Abbreviations**

346 **AA** : arachidonic acid; **2-AcG** : 2-acylglycerol; **AEA** : anandamide; **2-AG** : 2-arachidonoylglycerol;
347 **ALA** : alpha-linolenic acid; **AMPK** : AMP-activated protein kinase; **CNR1** : cannabinoid receptor 1;
348 **CNR2** : cannabinoid receptor 2; **DAGLA** : diacylglycerol lipase α ; **DAGLB** : diacylglycerol lipase β ;
349 **DPA** : docosapentaenoic acid; **DHA** : docosahexaenoic acid; **eCB** : endocannabinoid; **eCB-like** :
350 endocannabinoid-like compound; **ECS** : endocannabinoid system; **EPA** : eicosapentaenoic acid;
351 **FA** : fatty acid; **FAAH** : fatty acid amide hydrolase; **GPR55** : G protein-coupled receptor 55;
352 **GPR119** : G protein-coupled receptor 119; **LA** : linoleic acid; **MGLL** : monoacylglycerol lipase;
353 **mTOR** : mammalian target of rapamycin; **MUFA** : monounsaturated fatty acid; **NAE** : N-acyl-
354 phosphatidylethanolamine; **NAPEPLD** : N-acyl-phosphatidylethanolamine-hydrolyzing
355 phospholipase D; **OA** : oleic acid; **OEA** : N-oleoylethanolamide; **PA** : palmitic acid; **PDH** : pyruvate
356 dehydrogenase; **PDHA1** : pyruvate dehydrogenase E1 α 1 subunit; **PDHAB** : pyruvate
357 dehydrogenase E1 β subunit; **PDK4** : pyruvate dehydrogenase kinase 4; **PPARA** : peroxisome
358 proliferator-activated receptor α ; **PUFA** : polyunsaturated fatty acid; **SA** : stearic acid; **SBA** :
359 summer bear adipose tissue; **SBM** : summer bear muscle; **SBP** : summer bear plasma; **SBS** :
360 summer bear serum; **SFA** : saturated fatty acid; **WAT** : white adipose tissue; **WBA** : winter bear
361 adipose tissue; **WBM** : winter bear muscle; **WBP** : winter bear plasma; **WBS** : winter bear serum.

363

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531

532 **Authors' contributions**

533 EL, and FB conceived the study; CB, LCU, CD, JPB, IC, NBH, PD, AE, JMA, SB, EL, and FB performed
534 the experiments and analyzed the data; CB, FB and EL wrote the original draft; LCo, GG-K, CS, JS,
535 FB, and EL reviewed and edited the manuscript. All authors read and approved the final
536 manuscript.

537 **Ethics approval**

538 All samples and data were collected under protocols approved by the Swedish Ethical Committee
539 on Animal Experiment (applications Dnr C3/2016 and Dnr C18/2015), the Swedish Environmental
540 Protection Agency (NV-00741-18), and the Swedish Board of Agriculture (Dnr 5.2.18-3060/17).
541 All procedures complied with Swedish laws and regulations.

542

543 **Availability of data and materials**

544 The datasets generated during and/or analysed during the current study available from the
545 corresponding author on reasonable request.

546

547 **Competing interests**

548 The authors declare no competing interests.

549

550 **Figure legends**

551 **Figure 1: Lipidomics from summer and winter brown bear serum**

552 The six winter and summer bear serum mixes were prepared as described (Supplementary Table
553 S1). A: Total fatty acids (FAs) concentration. B: Total n-6 and n-3 FAs relative proportions of total
554 lipids. C: Detailed FAs relative proportions of total lipids. D: Molar ratios of EPA/AA and DHA/AA
555 in both summer and winter serum. Detailed lipidomic results are presented in Supplementary
556 Tables S2 and S3. Data are expressed in mmol/L for total FAs concentration, or molar percentage
557 of total lipids and are represented as mean \pm SEM of separate extractions and quantifications
558 from the six bear serum mixes (§: for EPA data are from three mixes only). * indicates p value
559 <0.05 when comparing seasons (** for $p<0.01$ and *** for $p<0.001$). AA:arachidonic acid, ALA:
560 alpha-linolenic acid, DHA: docosahexaenoic acid, DPA: docosapentaenoic acid, EPA:
561 eicosapentaenoic acid, LA: linoleic acid, OA: oleic acid, PA: palmitic acid, SA: stearic acid, SBS:
562 summer bear serum, WBS: winter bear serum.

563 **Figure 2: Circulating endocannabinoids concentration in brown bear plasma.** Concentration of
564 three major endocannabinoids compounds in bear plasma. Plasma were collected from bears at
565 both winter hibernating and summer active time points (Supplementary Table 1). Data are
566 expressed in ng/mL and are represented as mean \pm SEM of separate extractions and
567 quantifications from samples of individual animals (n=8). * indicates p value <0.05 when
568 comparing seasons (*** for $p<0.001$). AEA: anandamide, 2-AG: 2-arachidonoylglycérol, eCBs:
569 endocannabinoids, OEA: N-oleoylethanolamine, SBP: summer bear plasma, WBP: winter bear
570 plasma.

571 **Figure 3: Endocannabinoids concentration in brown bear muscle and adipose tissue.**

572 Concentration of three major endocannabinoids compounds in bear muscle and adipose tissue.
573 Tissues were collected from bears at both winter hibernating and summer active time points
574 (Supplementary Table 1). Data are expressed in pg/mg for muscle and adipose tissue
575 concentration and are represented as mean \pm SEM of separate extractions and quantifications
576 from samples of individual animals (n=5 for muscle, n=6 for adipose tissue). *** indicates p value
577 <0.0001 when comparing seasons. AEA: anandamide, 2-AG: 2-arachidonoylglycérol, OEA: N-

578 oleoylethanolamine, SBA: summer bear adipose tissue, SBM: summer bear muscle, WBA: winter
579 bear adipose tissue, WBM: winter bear muscle.

580

581 **Figure 4: Fold change in gene expression of target genes involved in endocannabinoids**
582 **biosynthesis and catabolism in brown bear muscle tissue.** Muscle tissues were collected from
583 bears at both winter hibernating and summer active time points (Supplementary Table 1), total
584 RNA was extracted and expression levels were measured by RT-qPCR. Data are normalized
585 against TBP mRNA levels and expressed as a fold change relative to the summer condition,
586 represented as mean \pm SEM of individual animals (n=8). * indicates p value <0.05 when
587 comparing seasons. CNR1: cannabinoid receptor 1, CNR2: cannabinoid receptor 2, DAGLA:
588 diacylglycerol lipase alpha, DAGLB: diacylglycerol lipase beta, FAAH: fatty acid amide hydrolase,
589 MGLL: monoacylglycerol lipase, NAPEPLD: N-acyl phosphatidylethanolamine phospholipase D,
590 SBM: summer bear muscle, WBM: winter bear muscle.