Specific shifts in the endocannabinoid system in hibernating brown bears

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Abstract

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

Background

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

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

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

Endogenous cannabinoids are involved in the regulation of many physiological processes, including thermogenesis, energy storage, metabolism, sleep cycles, circannual rhythms, stress response, feeding behavior, and skeletal muscle differentiation [20–23]. At the central level (e.g. hypothalamus), CNR1 is able to promote food intake and reduce energy expenditure [23,24]. In addition, CNR1 activation in adipose tissue leads to fatty acid and glucose uptake, and to upregulation of lipogenesis [23]. In liver, CNR1 signaling leads to increased expression of genes involved in the synthesis of fatty acids [25], and in skeletal muscle tissue, CNR1 activation triggers a decrease in glucose uptake and insulin sensitivity [23]. By contrast, OEA promotes lipolysis, fatty acid oxidation in skeletal muscle and liver, and it triggers an anorexigenic signal, notably through the nuclear receptor PPAR-α [26,27]. Considering the pleiotropic roles of ECS in neuronal signaling, regulation of behavior, appetite, energy metabolism, and circannual rhythms, it is not surprising to see important changes during hibernation. A decrease in ECS tone has notably been observed in hibernating marmots (Marmota monax and flaviventris) and ground squirrels (Spermophilus richardsonii) [21,28,29]. We hypothesized that a similar decrease could occur in hibernating bears, although specific changes due to their unique features during hibernation (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.

Results

Seasonal differences in serum lipids

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

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

Changes in plasma endocannabinoids and endocannabinoids-like compounds

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

Changes in endocannabinoid concentrations in muscle and adipose tissues

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

Changes in endocannabinoid pathway-related gene expressions in muscle tissue

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

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

Discussion

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

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

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

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

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

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

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

In muscle tissue, CNR1 signaling is involved in the regulation of metabolism key actors, like AMP-activated protein kinase (AMPK) and pyruvate dehydrogenase kinase 4 (PDK4) [45]. AMPK is activated during nutrient deprivation and hypoxia and modulates cell metabolism, enhancing both glucose transport and β-oxidation of fatty acids [46]. PDK4 is a major regulator of the pyruvate dehydrogenase (PDH) that controls the flux of carbohydrates into the TCA cycle and thus the switch between fatty acid and glucose oxidation [47]. In nonhibernating mammals, CNR1 activation leads to higher expression of the AMPKα1 subunit and a decrease of PDK4 expression that in turn enhances PDH activity [23,45,48,49]. Interestingly, recent studies have shown that PDK4 is upregulated in hibernating bear skeletal muscle [10,50], whereas A1 and B subunits of PDH are downregulated [10], and AMPK activity is reduced [51]. In hibernating bear muscle, AMPK and PDK4 expressions appear thus to be disconnected from direct regulation by CNR1.

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

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

Conclusions

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

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

Methods

Bear sample collection

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

Lipid extraction and analysis

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

transmethylated. Briefly, samples were treated with toluene-methanol (1:1, v/v) and boron trifluoride in methanol (14%). Transmethylation was carried out at 100 °C for 90 min in screw-capped tubes. Then 1.5 mL K_2CO_3 in 10% water was added and the resulting fatty acid methyl esters were extracted by 2 mL of isooctane and analyzed by gas chromatography (GC) with a HP6890 instrument equipped with a fused silica capillary BPX70 SGE column (60 x 0.25 mm). The vector gas was hydrogen. Temperatures of the Ross injector and the flame ionization detector were set to 230 °C and 250 °C, respectively. Data were expressed in mmol/L for total or individual fatty acids (FAs) concentration or molar percentage of total lipids for individual FAs. Detailed lipidomic results are presented in supplementary Table S2 (serum fatty acids concentrations) and S3 (serum fatty acids relative proportions).

Endocannabinoid quantification

Standard endocannabinoids (eCBs), i.e.- PEA, PEA-d5, OEA, OEA-d4, AEA, AEA-d4, 2AG, and 2AG-d5, were purchased from Cayman (Bertin BioReagent, Saint-Quentin en Yvelines, France). Mass spectrometry quality grade solvents were purchased from Fischer Scientific (Illkirch, France). Tissue samples (adipose and muscle tissues); c.a 100 mg) were crushed in an Omni Bead Ruptor 24 apparatus (Omni International, Kennesaw, USA) with circa twenty 1.4 mm OD zirconium oxide 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 containing 20 ng of PEA-d5, 2 ng OEA-d4, 10 ng AEA-d4, and 20 ng 2AG-d5. Then, each homogenate was added with 2 mL of $CHCl_3$/MeOH (1:1, v/v) and 500 µL of Tris (50 mM, pH=8), vortexed and centrifuged 10 min at 3000g. The organic layer was recovered and the upper aqueous phase was extracted twice with chloroform (1mL). Finally, organic phases were pooled and evaporated under vacuum.

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

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

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

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

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

Quantification of mRNAs by real-time RT-PCR

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

Statistical analysis

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

Abbreviations

AA : arachidonic acid; **2-AcG** : 2-acylglycerol; **AEA** : anandamide; **2-AG** : 2-arachidonoylglycerol; **ALA** : alpha-linolenic acid; **AMPK** : AMP-activated protein kinase; **CNR1** : cannabinoid receptor 1; **CNR2** : cannabinoid receptor 2; **DAGLA** : diacylglycerol lipase α; **DAGLB** : dicacylglycerol lipase β; **DPA** : docosapentaenoic acid; **DHA** : docosahexaenoic acid; **eCB** : endocannabinoid; **eCB-like** : endocannabinoid-like compound; **ECS** : endocannabinoid system; **EPA** : eicosapentaenoic acid; **FA** : fatty acid; **FAAH** : fatty acid amide hydrolase; **GPR55** : G protein-coupled receptor 55; **GPR119** : G protein-coupled receptor 119; **LA** : linoleic acid; **MGLL** : monoacylglycerol lipase; **mTOR** : mammalian target of rapamycin; **MUFA** : monounsaturated fatty acid; **NAE** : N-acyl-phosphatidylethanolamine; **NAPEPLD** : N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D; **OA** : oleic acid; **OEA** : N-oleoylethanolamide; **PA** : palmitic acid; **PDH** : pyruvate dehydrogenase; **PDHA1** : pyruvate dehydrogenase E1 α1 subunit; **PDHAB** : pyruvate dehydrogenase E1 β subunit; **PDK4** : pyruvate dehydrogenase kinase 4; **PPARA** : peroxisome proliferator-activated receptor α; **PUFA** : polyunsaturated fatty acid; **SA** : stearic acid; **SBA** : summer bear adipose tissue; **SBM** : summer bear muscle; **SBP** : summer bear plasma; **SBS** : summer bear serum; **SFA** : saturated fatty acid; **WAT** : white adipose tissue; **WBA** : winter bear adipose tissue; **WBM** : winter bear muscle; **WBP** : winter bear plasma; **WBS** : winter bear serum.

References

1. Carey HV, Andrews MT, Martin SL. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. Physiol Rev. 2003;83:1153–81.

2. Geiser F. Hibernation. Curr Biol. 2013;23:R188–93.

3. Folk GE, Hunt JM, Folk MA. Further Evidence for Hibernation of Bears. Bears Their Biol Manag. 1980;4:43.

4. Hellgren EC. Physiology of Hibernation in Bears. Ursus. 1998;10:467–77.

5. Hissa R, Hohtola E, Tuomala-Saramaki T, Laine T, Kallio H. Seasonal changes in fatty acids and leptin contents in the plasma of the European brown bear (Ursus arctos arctos). Ann Zool Fenn. 1998;35:215–24.

6. Manchi S, Swenson JE. Denning behaviour of Scandinavian brown bears Ursus arctos. Wildl Biol. 2005;11:123–32.

7. Toien O, Blake J, Edgar DM, Grahn DA, Heller HC, Barnes BM. Hibernation in black bears: independence of metabolic suppression from body temperature. Science. 2011;331:906–9.

8. LeBlanc PJ, Obbard M, Battersby BJ, Felskie AK, Brown L, Wright PA, et al. Correlations of plasma lipid metabolites with hibernation and lactation in wild black bears Ursus americanus. J Comp Physiol B. 2001;171:327–34.

9. Giroud S, Chery I, Bertile F, Bertrand-Michel J, Tascher G, Gauquelin-Koch G, et al. Lipidomics reveal seasonal shifts in large-bodied hibernator, the brown bear. Front Physiol [Internet]. 2019 [cited 2019 Mar 22];10. Available from: https://www.frontiersin.org/articles/10.3389/fphys.2019.00389/abstract

10. Chazarin B, Storey KB, Ziemianin A, Chanon S, Plumel M, Chery I, et al. Metabolic reprogramming involving glycolysis in the hibernating brown bear skeletal muscle. Front Zool. 2019;16:12.

11. Aloia RC, Raison JK. Membrane function in mammalian hibernation. Biochim Biophys Acta BBA - Rev Biomembr. 1989;988:123–46.

12. Munro D, Thomas DW. The role of polyunsaturated fatty acids in the expression of torpor by mammals: a review. Zool Jena. 2004;107:29–48.

13. Ruf T, Arnold W. Effects of polyunsaturated fatty acids on hibernation and torpor: a review and hypothesis. Am J Physiol-Regul Integr Comp Physiol. American Physiological Society; 2008;294:R1044–52.

14. De Petrocellis L, Di Marzo V. An introduction to the endocannabinoid system: from the early to the latest concepts. Best Pract Res Clin Endocrinol Metab. 2009;23:1–15.

15. Battista N, Di Tommaso M, Bari M, Maccarrone M. The endocannabinoid system: an overview. Front Behav Neurosci [Internet]. 2012 [cited 2019 Oct 28];6. Available from: http://journal.frontiersin.org/article/10.3389/fnbeh.2012.00009/abstract

16. Ueda N, Tsuboi K, Uyama T. Metabolism of endocannabinoids and related N-acylethanolamines: canonical and alternative pathways. FEBS J. 2013;280:1874–94.

17. Fezza F, Bari M, Florio R, Talamonti E, Feole M, Maccarrone M. Endocannabinoids, related compounds and their metabolic routes. Mol Basel Switz. 2014;19:17078–106.

18. Aizpurua-Olaizola O, Elezgarai I, Rico-Barrio I, Zarandona I, Etxebarria N, Usobiaga A. Targeting the endocannabinoid system: future therapeutic strategies. Drug Discov Today. 2017;22:105–10.

19. Di Marzo V. New approaches and challenges to targeting the endocannabinoid system. Nat Rev Drug Discov. 2018;17:623–39.

20. Ligresti A, Petrosino S, Di Marzo V. From endocannabinoid profiling to 'endocannabinoid therapeutics.' Curr Opin Chem Biol. 2009;13:321–31.

21. Vaughn LK, Denning G, Stuhr KL, de Wit H, Hill MN, Hillard CJ. Endocannabinoid signalling: has it got rhythm? Br J Pharmacol. 2010;160:530–43.

22. Iannotti FA, Silvestri C, Mazzarella E, Martella A, Calvigioni D, Piscitelli F, et al. The endocannabinoid 2-AG controls skeletal muscle cell differentiation via CB1 receptor-dependent inhibition of Kv7 channels. Proc Natl Acad Sci. 2014;111:E2472–81.

23. Piazza PV, Cota D, Marsicano G. The CB1 Receptor as the Cornerstone of Exostasis. Neuron. 2017;93:1252–74.

24. Cardinal P, Bellocchio L, Clark S, Cannich A, Klugmann M, Lutz B, et al. Hypothalamic CB1 cannabinoid receptors regulate energy balance in mice. Endocrinology. 2012;153:4136–43.

25. Osei-Hyiaman D, DePetrillo M, Pacher P, Liu J, Radaeva S, Bátkai S, et al. Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. J Clin Invest. 2005;115:1298–305.

26. Guzmán M, Verme JL, Fu J, Oveisi F, Blázquez C, Piomelli D. Oleoylethanolamide Stimulates Lipolysis by Activating the Nuclear Receptor Peroxisome Proliferator-activated Receptor α (PPAR-α). J Biol Chem. 2004;279:27849–54.

27. Bowen KJ, Kris-Etherton PM, Shearer GC, West SG, Reddivari L, Jones PJH. Oleic acid-derived oleoylethanolamide: A nutritional science perspective. Prog Lipid Res. 2017;67:1–15.

28. Stewart JM, Boudreau NM, Blakely JA, Storey KB. A comparison of oleamide in the brains of hibernating and non-hibernating Richardson's ground squirrel (Spermophilus richardsonii) and its inability to bind to brain fatty acid binding protein. J Therm Biol. 2002;27:309–15.

29. Mulawa EA, Kirkwood JS, Wolfe LM, Wojda SJ, Prenni JE, Florant GL, et al. Seasonal Changes in Endocannabinoid Concentrations between Active and Hibernating Marmots (Marmota flaviventris). J Biol Rhythms. 2018;33:388–401.

30. Græsli AR, Evans AL, Fahlman Å, Bertelsen MF, Blanc S, Arnemo JM. Seasonal variation in haematological and biochemical variables in free-ranging subadult brown bears (Ursus arctos) in Sweden. BMC Vet Res. 2015;11:301.

31. Deval C, Capel F, Laillet B, Polge C, Béchet D, Taillandier D, et al. Docosahexaenoic acid-supplementation prior to fasting prevents muscle atrophy in mice. J Cachexia Sarcopenia Muscle. 2016;7:587–603.

32. McGlory C, Calder PC, Nunes EA. The Influence of Omega-3 Fatty Acids on Skeletal Muscle Protein Turnover in Health, Disuse, and Disease. Front Nutr [Internet]. 2019 [cited 2019 Nov 12];6. Available from: https://www.frontiersin.org/articles/10.3389/fnut.2019.00144/full

33. Wei K, Piecewicz SM, McGinnis LM, Taniguchi CM, Wiegand SJ, Anderson K, et al. A liver Hif-2α-Irs2 pathway sensitizes hepatic insulin signaling and is modulated by Vegf inhibition. Nat Med. 2013;19:1331–7.

34. Schwartz GJ, Fu J, Astarita G, Li X, Gaetani S, Campolongo P, et al. The Lipid Messenger OEA Links Dietary Fat Intake to Satiety. Cell Metab. 2008;8:281–8.

35. Naughton SS, Mathai ML, Hryciw DH, McAinch AJ. Fatty Acid Modulation of the Endocannabinoid System and the Effect on Food Intake and Metabolism. Int J Endocrinol [Internet]. 2013 [cited 2019 Oct 24];2013. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3677644/

36. Walker CG, West AL, Browning LM, Madden J, Gambell JM, Jebb SA, et al. The Pattern of Fatty Acids Displaced by EPA and DHA Following 12 Months Supplementation Varies between Blood Cell and Plasma Fractions. Nutrients. 2015;7:6281–93.

37. Alvheim AR, Torstensen BE, Lin YH, Lillefosse HH, Lock E-J, Madsen L, et al. Dietary Linoleic Acid Elevates the Endocannabinoids 2-AG and Anandamide and Promotes Weight Gain in Mice Fed a Low Fat Diet. Lipids. 2014;49:59–69.

38. Alvheim AR, Malde MK, Osei-Hyiaman D, Lin YH, Pawlosky RJ, Madsen L, et al. Dietary linoleic acid elevates endogenous 2-AG and anandamide and induces obesity. Obes Silver Spring Md. 2012;20:1984–94.

39. Ghosh S, O'Connell JF, Carlson OD, González-Mariscal I, Kim Y, Moaddel R, et al. Linoleic acid in diets of mice increases total endocannabinoid levels in bowel and liver: modification by dietary glucose. Obes Sci Pract. 2019;5:383–94.

40. Matias I, Bisogno T, Marzo VD. Endogenous cannabinoids in the brain and peripheral tissues: regulation of their levels and control of food intake. Int J Obes. Nature Publishing Group; 2006;30:S7–12.

41. Matias I, Carta G, Murru E, Petrosino S, Banni S, Di Marzo V. Effect of polyunsaturated fatty acids on endocannabinoid and N-acyl-ethanolamine levels in mouse adipocytes. Biochim Biophys Acta BBA - Mol Cell Biol Lipids. 2008;1781:52–60.

42. DiPatrizio NV, Igarashi M, Narayanaswami V, Murray C, Gancayco J, Russell A, et al. Fasting stimulates 2-AG biosynthesis in the small intestine: role of cholinergic pathways. Am J Physiol - Regul Integr Comp Physiol. 2015;309:R805–13.

43. Fu J, Astarita G, Gaetani S, Kim J, Cravatt BF, Mackie K, et al. Food Intake Regulates Oleoylethanolamide Formation and Degradation in the Proximal Small Intestine. J Biol Chem. 2007;282:1518–28.

44. Izzo AA, Piscitelli F, Capasso R, Marini P, Cristino L, Petrosino S, et al. Basal and fasting/refeeding-regulated tissue levels of endogenous PPAR-alpha ligands in Zucker rats. Obes Silver Spring Md. 2010;18:55–62.

45. Watkins BA, Hutchins H, Li Y, Seifert MF. The endocannabinoid signaling system: a marriage of PUFA and musculoskeletal health. J Nutr Biochem. 2010;21:1141–52.

46. Kemp BE, Mitchelhill KI, Stapleton D, Michell BJ, Chen Z-P, Witters LA. Dealing with energy demand: the AMP-activated protein kinase. Trends Biochem Sci. 1999;24:22–5.

47. Zhang S, Hulver MW, McMillan RP, Cline MA, Gilbert ER. The pivotal role of pyruvate dehydrogenase kinases in metabolic flexibility. Nutr Metab. 2014;11:10.

48. Cavuoto P, McAinch AJ, Hatzinikolas G, Cameron-Smith D, Wittert GA. Effects of cannabinoid receptors on skeletal muscle oxidative pathways. Mol Cell Endocrinol. 2007;267:63–9.

49. Esposito I, Proto MC, Gazzerro P, Laezza C, Miele C, Alberobello AT, et al. The Cannabinoid CB1 Receptor Antagonist Rimonabant Stimulates 2-Deoxyglucose Uptake in Skeletal Muscle Cells by Regulating the Expression of Phosphatidylinositol-3-kinase. Mol Pharmacol. 2008;74:1678–86.

50. Mugahid DA, Sengul TG, You X, Wang Y, Steil L, Bergmann N, et al. Proteomic and Transcriptomic Changes in Hibernating Grizzly Bears Reveal Metabolic and Signaling Pathways that Protect against Muscle Atrophy. Sci Rep. 2019;9:19976.

51. Vella CA, Nelson OL, Jansen HT, Robbins CT, Jensen AE, Constantinescu S, et al. Regulation of metabolism during hibernation in brown bears (Ursus arctos): Involvement of cortisol, PGC-1α and AMPK in adipose tissue and skeletal muscle. Comp Biochem Physiol A Mol Integr Physiol. 2019;110591.

52. Proulx K, Cota D, Castañeda TR, Tschöp MH, D'Alessio DA, Tso P, et al. Mechanisms of oleoylethanolamide-induced changes in feeding behavior and motor activity. Am J Physiol-Regul Integr Comp Physiol. 2005;289:R729–37.

53. Sarro-Ramirez A, Sanchez-Lopez D, Tejeda-Padron A, Frias C, Zaldivar- Rae J, Murillo-Rodriguez E. Brain Molecules and Appetite: The Case of Oleoylethanolamide. Cent Nerv Syst Agents Med Chem. 2013;13:88–91.

54. Fedele S, Arnold M, Krieger J-P, Wolfstädter B, Meyer U, Langhans W, et al. Oleoylethanolamide-induced anorexia in rats is associated with locomotor impairment. Physiol Rep. 2018;6:e13517.

55. Murillo-Rodríguez E, Palomero-Rivero M, Millán-Aldaco D, Arias-Carrión O, Drucker-Colín R. Administration of URB597, Oleoylethanolamide or Palmitoylethanolamide Increases Waking and Dopamine in Rats. PLOS ONE. 2011;6:e20766.

56. Swenson JE, Sandegren F, Brunberg S, Wabakken P. Winter den abandonment by brown bears Ursus arctos: causes and consequences. Wildl Biol. Nordic Board for Wildlife Research; 1997;3:35–8.

57. Linnell JDC, Swenson JE, Andersen R, Barnes B. How Vulnerable Are Denning Bears to Disturbance? Wildl Soc Bull. 2000;28:400–13.

58. Evans AL, Singh NJ, Fuchs B, Blanc S, Friebe A, Laske TG, et al. Physiological reactions to capture in hibernating brown bears. Conserv Physiol [Internet]. 2016 [cited 2020 Feb 9];4. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5156896/

59. Chanon S, Chazarin B, Toubhans B, Durand C, Chery I, Robert M, et al. Proteolysis inhibition by hibernating bear serum leads to increased protein content in human muscle cells. Sci Rep. 2018;8:5525.

60. Lefils J, Géloën A, Vidal H, Lagarde M, Bernoud-Hubac N. Dietary DHA: time course of tissue uptake and effects on cytokine secretion in mice. Br J Nutr. 2010;104:1304–12.

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Authors' contributions

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

Ethics approval

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

Availability of data and materials

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

Competing interests

The authors declare no competing interests.

Figure legends

Figure 1: Lipidomics from summer and winter brown bear serum

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

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

Figure 3: Endocannabinoids concentration in brown bear muscle and adipose tissue. Concentration of three major endocannabinoids compounds in bear muscle and adipose tissue. Tissues were collected from bears at both winter hibernating and summer active time points (Supplementary Table 1). Data are expressed in pg/mg for muscle and adipose tissue concentration and are represented as mean ± SEM of separate extractions and quantifications from samples of individual animals (n=5 for muscle, n=6 for adipose tissue). *** indicates p value <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 ± 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.