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Biochemical Foundations of Health and Energy Conservation in Hibernating Free-Ranging Subadult Brown Bear Ursus arctos

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ABSTRACT

Brown bears (Ursus arctos) hibernate for 5-7 months without eating, drinking, urinating and defecating at a metabolic rate of only 25% of the summer activity rate. Nonetheless, they emerge healthy and alert in spring. We quantified the biochemical adaptations for hibernation by comparing the proteome, metabolome, and hematologic features of blood from hibernating and active free-ranging subadult brown bears with a focus on conservation of health and energy. We found that total plasma protein concentration increased during hibernation, even though the concentrations of most individual plasma proteins decreased, as did the white blood cell types. Strikingly, antimicrobial defense proteins increased in concentration. Central functions in hibernation involving the coagulation response and protease inhibition, as well as lipid transport and metabolism, were upheld by increased levels of very few key or broad-specificity proteins. The changes in coagulation factor levels matched the changes in activity measurements. A dramatic 45-fold increase in sex-hormone-binding-globulin SHBG levels during hibernation draws, for the first time, attention to its significant but unknown role in maintaining hibernation physiology. We propose that energy for the costly protein synthesis is reduced by three mechanisms, (i) dehydration, which increases protein concentration without de novo synthesis; (ii) reduced protein degradation rates due to a 6 °C reduction in body temperature, and decreased protease activity; and (iii) a marked redistribution of energy resources only increasing de novo synthesis of few key proteins. This comprehensive global data identified novel biochemical strategies for bear adaptations to the extreme condition of hibernation, and have
Hibernation is a physiological survival strategy when food is limited during winter. The ability to hibernate is an old evolutionary adaptation used by very different families of mammals. To date most physiological and molecular studies have been carried out in small hibernating experimental animals like rodents, bats, and hedgehogs, which however hibernate with body temperatures near the freezing point, and therefore must adapt differently from bears hibernating at > 30 °C. Despite a wealth of information on hibernation and torpor physiology, the trigger of the physiological adaptations remains elusive (1-3). Yet the trigger controls gene regulations, which are the primary driver of the hibernation phenotype (4).

Important physiological responses in hibernating bears include a 6 °C reduction in body temperature (5-7), a reduction to 24% in cardiac output (8) and to 25% in metabolic rate (oxygen consumption) compared with those of active bears (5), and increased affinity of hemoglobin to oxygen (9). Adaptations for hibernation overcome starvation, anuria, hyperlipidemia, and inactivity. At the same time, muscle and bone are preserved (10-13), and atherosclerosis (14) and cardiac disease (15, 8) appear to be absent. The bears remain both healthy and alert or easily aroused during hibernation. For these reasons, the hibernating bear is a fascinating model in the biological and medical science fields.

We wanted to elucidate the biochemical foundations leading to the important protection of health, and the dramatic energy saving in hibernating bears. As the bloodstream has primary functions in molecular and cellular defenses, in transport of nutrients, oxygen and wastes, and in signaling, the levels of blood constituents will reflect the conditions of the animal. Therefore, we have subjected paired winter and summer bear plasma samples to mass spectrometric based label-free quantitative proteome analyses, and to targeted metabolome analyses of phospholipids, hexose sugars, and amine-containing compounds, and to hematologic analyses, including functional coagulation factor analyses. Such quantitative analyses have not been carried out on a global scale before in bears, and provide the solid foundation for specific hypothesis and insights in the regulation of mammalian hibernation physiology.

We describe a unique sample set of seven free-ranging 2- to 3-year old brown bears, and an experimental design that involves minimal plasma handling. The use of sub-adult animals reduced the risk of interference of pregnancy, sexual activity, and past diseases in the analyses. We document that levels of innate antimicrobial proteins protecting health are significantly increased in hibernation compared to summer activity, when adapted immunity takes over. We propose that energy is conserved in hibernation by decreased protein synthesis, which is counteracted by dehydration and by a reduction in chemical and biochemical degradation rates. Furthermore, our data provide a significant and unexpected clue to the regulation of hibernation physiology by sex-hormone-binding-globulin (SHBG), the levels of which increased 45-fold in hibernation inactivity over summer activity.

Results

Data—Blood was collected from four female and three male free-ranging subadult anesthetized brown bears during hibernation in February 2010 and from the same seven animals when active in June 2010 (16). Similar paired blood samples collected in later years were used in validation analyses. For proteome analysis, we digested non-enriched, non-diluted blood plasma with trypsin in spin cups to minimize handling errors. Total digests were subjected to high-resolution quadrupole orbitrap mass spectrometry, to obtain label-free quantification of plasma proteins across four orders of magnitude of molar concentration. To determine the metabolic profiles of the samples, we used targeted mass spectrometry to measure 186 phospholipids, amino-containing components, and hexose sugars. A commercially available human reference plasma sample was analyzed in parallel. Analysis of each winter sample was immediately followed by that of the summer sample of the same bear (Supplemental Table S1, protein data; Supplemental Table S2, metabolite data; and Fig. 1). We denoted bear proteins by the most similar human gene names, and the corresponding UniProt consortium accession numbers and function assignments (Supplemental Table S1). The bear protein database we used (Supplemental file bear-protein-db-Febr2013.txt) was derived from the
genome sequence of polar bear *Ursus maritimus* (17), and annotated by BlastP against the non-redundant human NCBI Reference Sequence Database. These data were complemented by standard clinical data (Supplemental Table S3), some of which have been published previously (6, 14, 18), and by quantitative activity analyses of seven coagulation factors (Supplemental Table S4). We calculated winter/summer (W/S) ratios of protein and metabolite levels and blood cell counts for each individual bear, after which we calculated the mean and the *p*-value from paired *t*-tests for each factor. Only *p*-values > 0.05 are reported below to indicate a lower significance of the corresponding W/S. We observed no differences between males and females or between the 2-year old bear and the six 3-year old bears (Fig. 1B) reinforcing our conclusion that they are subadult animals (19). We found very few intracellular proteins released from bone and muscle tissues (Supplemental Table S1, sheet Cells&others). Therefore, these are not discussed further.

**Plasma Protein and Hemoglobin Concentrations**—UV and UV-VIS absorption spectra of bear plasma samples showed clear differences between the seven winter and summer samples (Fig. 1A). Protein and hemoglobin concentrations derived from the A280 and A415 peaks are summarized in Supplemental Table S5. The protein concentration was significantly higher in 2010 winter plasma, 68.9 ± 5.1 g L⁻¹, than in summer plasma, 63.0 ± 4.6 g L⁻¹, corresponding to a 9.6% increase. For one bear we found similar protein concentrations in winter and summer. The significance of increased plasma protein concentration during hibernation was verified on paired samples collected in 2013 from another seven subadult bears. Again, winter plasma, with a concentration of 67.0 ± 2.2 g L⁻¹, was more concentrated than summer plasma, with a concentration of 54.0 ± 2.4 g L⁻¹, corresponding to an increase of 24.1% (Supplemental Table S5). It is notable that plasma concentration during hibernation was similar for the 2010 and 2013 samples, i.e. 67-69 g L⁻¹.

The hemoglobin spectra (Fig. 1A) showed fully oxygenated hemoglobin with the characteristic Soret peak at 415 nm, as predictable for plasma exposed to air. We found high and variable hemoglobin levels in all 2010 and 2013 plasma samples (Supplemental Table S5), most likely introduced by some hemolysis during transport on ice to centrifugation. The natural hemoglobin concentration in bear plasma therefore remains unknown and might be like that in healthy humans, in whom the range is 0.01 - 0.04 g L⁻¹. Protein concentrations in bears measured at A280 were affected < 1.4% by hemoglobin.

In Supplemental Table S1, proteins originating from red blood cell rupture are shown in italics. The hemoglobin alpha (HBA) and beta (HBB) chains showed an increase to 3-fold their summer levels in winter, which is in agreement with the hemoglobin concentration determined by absorption spectroscopy. The levels of peroxiredoxin-2 isoform (PRDX2), also originating from red blood cells, showed a similar increase to 4-fold its summer levels. Carbonic anhydrase 1 (CA1) data indicated an increase of the levels of this protein to 12-fold its summer levels, but this protein was only detected in five bears and with low protein scores; CA1 is known to be less abundant in red blood cells than hemoglobin and PRDX2. No proteins from red blood cells were increased in summer. Thus the independent hemoglobin absorption data provide an initial quality control of the quantitative proteomics data.

**Red Blood Cells, Hemoglobin and Iron Metabolism**—Blood and blood plasma appeared more viscous in winter than in summer, and the plasma yield was lower, which indicated a state of dehydration during hibernation. Red blood cell (RBC) count, total hemoglobin (HB) levels, and the hematocrit (volume of RBCs/volume of blood) during hibernation were 1.3-fold the summer levels (Supplemental Table S3). Total plasma protein and albumin (ALB) concentrations also increased significantly in hibernation (Fig. 1A top; Tables S5, S1), despite a decrease in the concentration of most individual plasma proteins (Fig. 1C top). RBC proteins were excluded from the analyses.

The levels of haptoglobin-2 (HP), which binds potentially harmful HB released naturally from RBC, were increased by a factor of 4.7 in winter. The HP-HB complex has high oxygen affinity, effectively blocking uncontrolled oxygen release and the creation of reactive oxygen species (20). HP permits proteolysis of globin chains and conserves iron as well as ceruloplasmin (CP, W/S ratio 1.6), which oxidizes Fe²⁺ to Fe³⁺. The levels of the Fe³⁺ transporter transferrin (TF has two forms both analyzed in this study), and those of the transferrin
receptor (TFRC, W/S ratio 0.71), which is required for iron recycling but not oxidation, were lower in winter. The levels of hemopexin (HPX, W/S ratio 0.74), which transports heme to the liver for breakdown and iron recovery, were similarly decreased in winter. Protection against oxidation and preservation of Fe^{3+} seems to be important in hibernation.

**Defenses and Immunity**—Invading pathogens are first met by an organism’s outer and inner surfaces (skin, lung, intestine) and challenged by antimicrobial peptides in epithelial cells. In hibernating bears, we detected raised levels of the circulating antimicrobial defense proteins CAMP, HP, LYZ, and PGLYRP2 (which increased in winter to 3.7-fold, 4.7-fold, 2.7-fold, and 2.2-fold of their levels in summer, respectively) (Supplemental Table S1). Furthermore, proteins protecting against self-proteins were increased or retained during hibernation, as the levels of serum-amylloid-P-component (APCS, W/S ratio 1.7), which scavenges material released from damaged blood cells like HP, increased and the levels seemed unchanged for clusterin that prevents aggregation of non-native proteins (CLU, W/S ratio 0.90, p = 0.10).

By contrast, both the innate and acquired cellular and humoral immune defenses were generally suppressed during hibernation. The white blood cell count (W/S ratio 0.6), and the numbers of the relatively short-lived neutrophils (W/S ratio 0.5), and monocytes (W/S ratio 0.6) were significantly lower, as reported previously (6), whereas the numbers of the more long-lived lymphocytes were unchanged (Supplemental Table S3). The significantly reduced levels of macrophage stimulatory protein MST1 (W/S ratio 0.20), and monocyte differentiation antigen CD14 (W/S ratio 0.22), which recognizes lipopolysaccharide on gram negative bacteria, emphasized the suppression of innate immunity in hibernation.

The lectin, classic, and alternative pathways of complement activation, as well as components of the membrane attack complex (MAC), were all represented in the proteome data (Fig. 2; Table S1). In the lectin and classic complement activating pathways, bacteria are bound by lectins and antibodies, respectively. The lectin-activating pathway is initiated by lectin FCN1 and protease MASP1, and the levels of these proteins were decreased during hibernation to 0.21-fold and 0.15-fold of the summer levels, respectively. The antibody-initiated classic pathway was also down-regulated, as indicated by decreases in the levels of subunits of complement protease C1 (C1QA, C1QB, C1R, and C1S, which had similar W/S ratios of 0.49, 0.43, 0.40, and 0.39, respectively), and its SERPING1 inhibitor (W/S ratio 0.61). C1, like MASP1 of the lectin pathway, cleaves C2 and C4 generating the active C3 convertase C4C2a. Subunit C1QG and complement C2 could not be identified because they were missing in the bear protein database.

The alternative pathway is initiated by the binding of complement factor B (CFB, W/S ratio 0.76) to hydrolyzed C3 (C3H2O), which is formed slowly by a spontaneous process and present at low levels in plasma. Proteolytic cleavage of CFB in this complex by complement factor D (CFD) produces the alternative pathway C3 convertase complex [(C3H2O)BbP, stabilized by properdin (P)]. CFD was absent from the bear protein database and was therefore not included in the analysis.

Initiation of the three complement pathways (Fig. 2) leads to the cleavage of their common substrate C3 (W/S ratio 0.89) followed by exposure of a reactive thiol ester. The C3b fragment covalently attaches to proximal molecules or cell surfaces and changes C3 convertase to C5 convertase. The MAC complex, which forms pores in cell membranes, is assembled of C5b; C6; C7; the C8A, C8B, C8G subunits of C8; and the C9 multimer (W/S ratios 0.77, 0.39, 0.80, 0.63, 0.65, 0.31, and 0.88, respectively). We conclude that the activity of all pathways of the complement system is significantly down-regulated in hibernation.

We also detected decreased levels of complement regulatory proteins CFHR2 (two dissimilar bear transcripts of complement factor H-related protein 2, with W/S ratios 0.44 and 0.25) in hibernating bears, whereas complement factor H (CFH) was not observed. Decreased levels of complement factor I (CFI, W/S ratio 0.68), were also detected in winter. CFHR2 competes with CFH, a cofactor of CFI, which degrades C3b and C4b. The subunits of C4BP, C4BPA (W/S ratio 1.07, p = 0.25) and C4BPB (W/S ratio 1.21, p = 0.075), as well as the target component C4 (W/S ratio 1.11, p = 0.16) showed non-significant changes in protein levels. C4BP functions in the protection of host cells proximal to an attacking pathogen in complex with partners including CFI and CFH, which explains its
conservation in hibernation. C-reactive protein (CRP, W/S ratio 0.66) is a prominent marker of inflammation and activates the complement system in complex with phosphocholine and C1Q. Vitronectin (VTN, W/S ratio 0.88), in complex with C5b, C6 and C7, inhibits attachment of the MAC complex to cell membranes. Taken together, these findings suggest that the observed changes in the levels of regulators of the complement pathways correspond with those of their target components.

In addition to the well-studied human complement components, we found a C3-like component (W/S ratio 0.45), which is ~40% identical in amino acid sequence to human C3. This component is present also in giant panda, dog, and pig, but not in human or mouse. In human C3, C4, and A2M proteins, the active site thiol-ester-forming sequences linking Cys and Gln are Cys-Gly-Glu-Gln. In C3-like proteins the homologous sequences are Cys-Pro-Glu-Gln. The larger and rigid proline residue may inhibit spontaneous thiol ester formation. However, our molecular modeling of this C3-like domain was inconclusive in this respect, and further analysis will be needed. Mass spectrometry data indicated C3-like plasma concentrations to be very low compared with that of C3. The function of C3-like component is unknown.

We attempted to distinguish the immunoglobulin classes IgM, IgA, IgG, IgE, and IgD, and light chain types IgL and IgK, via their constant regions. However, the automatic predictions of bear transcripts of the complex IGH, IGK and IGL loci appeared to be of poor quality. Our manually verified transcripts are included in Supplemental Table S1. The levels of IGHM, IGHG1, IGLC1, IGKC, and IGJ were reduced in hibernation to approximately 90%, whereas the levels of the secretory IGHA1 were doubled. IGHD and IGHE concentrations are normally low and these proteins were not detected. The levels of immunoglobulins varied among the bears, as did that of the lymphocytes producing them.

The levels of the immunoglobulin helper proteins VPREB1, PIGR, ATRN, B2M, and CD5L were reduced in hibernating bears (W/S ratios 0.54, 0.30, 0.70, 0.24, and 0.87, respectively). We included the alpha-1-microglobulin–bikunin precursor (AMBP, W/S ratios 0.83) in our analysis of immunity-related proteins because alpha-1-microglobulin inhibits immunological functions of white blood cells in vitro, and bikunin, which is called the light chain of inter-alpha-trypsin inhibitor, exhibits broad serine protease inhibitor activity in complex with the four heavy chains of the inhibitor (ITIH1, ITIH2, ITIH3, and ITIH4; W/S ratios 1.07, p = 0.2; 0.94, p = 0.2; 0.25; and 0.45, respectively). ITIH4 plays a role in the acute-phase reaction.

We conclude that the humoral and cellular immune responses were significantly reduced in hibernation and taken over by non-specific circulating antimicrobial defense proteins and secretory IGHA1, which had their levels raised by 2-fold to 5-fold.

Platelets, Coagulation and Fibrinolysis—Activated platelets attach to a site of tissue injury or to blood vessels, and to each other, to form aggregates. Platelet count was down to 0.67-fold of the summer levels in blood of hibernating bears (Supplemental Table S3). The levels of platelet-activating factor phospholipid 1-O-hexadecyl-2-acetyl-phosphatidylcholine are balanced by synthesis and the inactivation by platelet-activating factor acetylhydrolase (PLA2G7, W/S ratio 0.14) (Supplemental Table S1). The von Willebrand factor (VWF, W/S ratio 0.73) is essential in initial clotting, promoting the adhesion of platelets to the site of vascular injury by bridging the platelet receptor GP1BA and the sub-endothelial collagen matrix. Later in the coagulation cascade, VWF stabilizes coagulation factor VIII (F8). Thrombospondin (THBS1) is a multifunctional protein mediating cell-to-cell and cell-to-matrix interaction. THBS1 (W/S ratio 12, p = 0.08) and GP1BA (W/S ratio 1.8) were observed in the plasma of four bears only, indicating levels marginal to our detection. Histidine-rich glycoprotein (HRG, W/S ratio 0.14) binds a multitude of ligands, including THBS1. Both HRG and THBS1 interact with an abundance of biological partners, and the apparent increase in THBS1 could indicate a major role in other pathways.

The blood plasma coagulation cascade is initiated by two pathways that both activate factor X (F10, W/S ratio 1.2) leading to fibrin clot formation (Fig. 2). The intrinsic (contact activation) pathway begins with complex formation of kininogen (KNG1, W/S ratio 0.67), kallikrein (KLKB1, W/S ratio 0.58), and factor XII (F12, W/S ratio 0.86, p = 0.1) on collagen. This results in activation of F12, which cleaves F11 (not detected), which then
cleaves F9. F9 (W/S ratio 0.70), assisted by its cofactor F8 (not detected) cleaves F10. However, the role of F12, if any, in normal hemostasis is unclear as humans and animals totally deficient in F12 have no bleeding tendency. Kallistatin (SERPINA4, W/S ratio 0.49) inhibits kallikrein. Hence, the intrinsic pathway was significantly down-regulated in hibernation. The extrinsic, or tissue factor pathway components tissue factor (F3) and factor VII (F7) were not detected in winter or summer, which indicates low activity of this pathway. Fibrin coagulation is achieved by thrombin (F2, W/S ratio 1.3), which is activated from prothrombin by F10 and cofactor Va (F5, W/S ratio 0.98, \( p = 0.44 \)). Activated F5 and F8 are degraded by PROC protease (W/S ratio 0.80). Anticoagulant PROS assists PROC but the levels of PROS increased in winter to 1.5-fold the summer levels, which suggest PROS may have additional unknown roles.

Most remarkably, the levels of the ultimate clotting effector proteins F10 and F2, and those of the fibrinogen substrate subunits FGA (W/S ratio 1.1, \( p = 0.2 \)), FGB (W/S ratio 1.2, \( p = 0.12 \)), and FGG (W/S ratio 1.2, \( p = 0.13 \)) increased in winter, and to approximately the same extent. Indeed, the stoichiometric 2:2:2 ratios of fibrinogen subunits FGA, FGB, and FGG changed to the same extent in the seven individual bears; the non-significant \( p \)-values reflect that the W/S ratios among the bears varied between 0.8 and 2.6. The data show that the central clotting mechanism is fully active during hibernation. By contrast, all other components of fibrin clot formation and degradation had moderately decreased levels. Fibrin monomers are cross-linked by factor XIII, which is composed of subunits F13A1 (W/S ratio 0.80, \( p = 0.23 \)), and F13B (W/S ratio 0.52). Fibrin monomer degradation is reduced by carboxypeptidase B2 (CPB2, W/S ratio 0.50).

Plasmin is essential to fibrin clot hydrolysis (Fig. 2). Plasmin is activated from plasminogen (PLG, W/S ratio 0.81) by a number of proteases and has broad specificity and range of biological substrates in addition to fibrin. The major inhibitor of plasmin, alpha-2-antiplasmin (SERPINF2, W/S ratio 0.79), had a decrease similar to plasminogen. The serine protease hyaluronan-binding-protein 2 (HABP2, W/S ratio 0.67) is also implicated in fibrinolysis. As collagen is involved in clotting, we included the collagen degrading protease xaa-Pro dipeptidase (PEPD, W/S ratio 0.38) among the list of clot clean-up proteins (Supplemental Table S1). A number of specific serine protease inhibitors were down-regulated or had unchanged levels, including SERPINC1 (W/S ratio 0.56), SERPINA1 (W/S ratio 0.86), SERPING1 (W/S ratio 0.90, \( p = 0.13 \)), and SERPINA5 (W/S ratio 1.08, \( p = 0.19 \)). By contrast, levels of the non-specific abundant protease inhibitor alpha-2-macroglobulin (A2M) were significantly increased to 1.7-fold the summer levels, suggesting that A2M can substitute for the specialized serpins.

The spectacular regulated increases of only the key components of coagulation, F10, F2, and fibrinogen, demonstrated by mass spectrometry (MS), were verified and extended (F7, F8) by functional analyses of six factors and immunochemical analysis of VWF (Table 1; Fig. 2). The excellent agreements between MS counted factor levels and coagulation functionality demonstrated that molecules are active, and serves as mutual validations of the quantitative data.

### Table 1

<table>
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<th>Protein</th>
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<th>Functional data Mean W/S</th>
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**Lipid Transport and Metabolism**—In hibernating brown bears, energy and water are obtained from catabolism of lipids in adipose tissue, rather than via the digestive tract. Proteome, metabolome, and hematologic data demonstrated this marked switch (Tables S1, S2, S3). Most remarkable, lipase (CEL, W/S ratio 32), the carrier proteins serum albumin (ALB, W/S ratio 1.2), the apolipoproteins APOB...
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W/S ratio 1.3), APOC1 (W/S ratio 2.3), APOD (W/S ratio 1.7), and cholesterol (Chol) in form of the "bad" low-density lipoprotein (LDL) particles were significantly up-regulated in hibernation. CEL, which is almost exclusively expressed in the pancreas, is activated by bile salt. ALB transports free fatty acids to all cell types from adipocytes, where they are produced from triglycerides (W/S ratio 2.3). The increase in APOB levels in hibernation is in agreement with the increase in LDL particles in hibernation found by Arinell et al. (14), as each LDL particle contains one molecule of APOB. The significant increases in the winter levels of APOC1 and APOD, apolipoproteins that play important roles in Chol transfer, are in accordance with the significant increases in levels of total Chol (W/S ratio 1.5), and Chol-LDL (W/S ratio 2.6) during hibernation. The Chol from high-density lipoprotein particles (Chol-HDL) was not significantly different in hibernating and active bears (W/S ratio 0.97, p = 0.18; Supplemental Table S3). Phosphatidylcholine-sterol acyltransferase (LCAT, which produces cholesteryl esters) and phospholipid transfer protein (PLTP) interact with HDL, and both showed non-significant changes in their levels (LCAT W/S ratio 0.86, p = 0.21; PLPT W/S ratio 0.76, p = 0.21). Phosphatidylinositol-glycan-specific phospholipase D (GPLD1, W/S ratio 0.80) and serum paraoxonase/arylesterase 1 (PON1, W/S ratio 0.66) had significant but moderately lowered levels in hibernation.

The human chromosome 11q23 contains a gene cluster that encodes apolipoproteins APOA1 (W/S ratio 0.60), APOC3 (W/S ratio 0.75, p = 0.11), and APOA4 (W/S ratio 0.04). These proteins were all down-regulated in hibernation. The very low levels of APOA4 could indicate an active degradation. APOA1 is the major protein component of HDL in plasma, which also contains APOA4. The levels of proteins that interact with HDL were all decreased in winter to ~0.7-fold their summer levels, suggesting that HDL levels might be similarly reduced despite the non-significant change in Chol-HDL levels. A down-regulation of APOC3 would be in accord with the function of this apolipoprotein as an inhibitor of lipoprotein and hepatic lipases (LPL, LIPC), as higher activity of these lipases is essential to the hydrolysis of triglycerides in hibernation. APOC2, APOA5, and APOF levels were below the limit of detection of the proteome approach, and the APOE and apolipoprotein(a) (LPA) sequences were not present in the bear protein database. The levels of individual lipases, including LPL and LIPC, were also below the limits of detection, whereas the levels of CEL, a general lipase that hydrolyzes both triglycerides and sterol esters, increased in winter to 32-fold the summer levels, which is consistent with a greater than 10-fold total lipase activity increase in blood plasma during bear hibernation (Supplemental Table S3).

It is noteworthy that the increased activity in liver and adipose tissues in hibernation did not result in detectable levels of released intracellular proteins from exhausted cells of these tissues.

In contrast with proteins, which normally require an active vesicular transport out of cells, we consider the concentrations of intracellular metabolites to be reflected in their blood plasma levels. We characterized the extreme changes in metabolic conditions in hibernation by targeted mass spectrometry quantifying 40 carnitines; 90 phosphatidylcholines, including lysoPC with one acyl group, PCaa with two acyl groups, and PCae with one acyl and one alkyl ether group; 15 sphingomyelins; 21 amino acids; 19 biogenic amines; and hexose sugars in plasma of hibernating and active bears and a human plasma reference sample (Supplemental Table S2; and Fig. 1B, C). Soluble metabolites need no transporter proteins, and we found none in the bear proteome for carnitines, amino acids, biogenic amines, or hexoses.

Hexose sugars (predominantly glucose, but also fructose, mannose, galactose and others that have identical masses) levels covered a broad range and were increased moderately in hibernation. The mean hexose levels in winter and summer bear samples, and in the human reference were 10.0 mM, 8.9 mM, and 8.2 mM, respectively. This is notably higher than solely glucose measured in enzymatic assays of bear samples (18) (Supplemental Table S2).

Carnitines are required in all tissues for transport of fatty acids from the cytosol to the mitochondria for fatty acid oxidation. All were up-regulated in hibernation, in accordance with the essential catabolism of lipids that occurs during this state. Sum concentration of all carnitines in winter and summer bear samples, and in human samples were 95 µM, 56 µM, and 61 µM, respectively.

Phosphatidylcholines (PC), along with cholesterol, are essential constituents of cell plasma
membranes. The levels of lysoPC, the products of hydrolysis of PCaa, were increased by a factor ~1.7 in hibernation, which indicates increased cell membrane turnover, a probable result of shrinking adipocytes as lipid stores are depleted. The sum concentrations of lysoPC of hibernating bears and human were similar, 265 µM and 251 µM, respectively. PCaa is the dominant phospholipid type in bear plasma and its levels increased (W/S ratio ~1.2), whereas the levels of PCae decreased (W/S ratio ~0.8) on average in hibernation. Concentrations of individual PCaa and PCae in bear followed the pattern of the corresponding concentrations of these products in the human sample. However, the total concentrations in bear plasma were 2.6-fold to 4.6-fold those in human. The concentrations of total PCaa in hibernation, summer, and human samples were 4.3 mM, 3.6 mM, and 1.4 mM, respectively. The total PCae concentrations in winter, summer, and human were lower: 664 µM, 846 µM, and 185 µM, respectively.

Sphingomyelins (SM) are components of the cell membrane that are thought to increase chemical resistance. SM levels were similar in hibernating and active bears. However, they were 2- to 5-fold higher than the levels of these metabolites in human plasma. Total sphingomyelin concentrations in winter, summer, and in the human sample were 745 µM, 710 µM, and 257 µM, respectively. Similarly, the levels of SM-elevating sphingomyelinase-like-phosphodiesterase-3a (SMPDL3A, W/S ratio 0.95, p = 0.34) seemed to be unchanged in hibernation.

The ratio of total unsaturated/total saturated plasma phospholipids (PC plus SM) was unchanged in hibernation, which suggested that cell membrane fluidity was not adjusted to the 6 °C temperature decrease in hibernation.

Nitrogen Metabolism—It is a major challenge to the physiological adaptations for hibernation in bears to conserve and recycle nitrogen in protein metabolism within the metabolic energy available in this condition, while avoiding intoxication. We analyzed amino acids and biogenic amines as phenylisothiocyanate derivatives by targeted mass spectrometry and found the plasma concentrations of these factors in bear were mostly within 50% of the reference range for humans (Supplemental Table S2). Significant physiological differences included up-regulation of molecules containing several nitrogen atoms during hibernation. These molecules included creatinine [W/S ratio 3.9 determined by mass spectrometry (Supplemental Table S2); W/S ratio 3.8 determined by standard clinical analysis (Supplemental Table S3)], ornithine (W/S ratio 3.6), acetyl-ornithine (W/S ratio 1.9), lysine (W/S ratio 2.0), citrulline (W/S ratio 2.6), glutamine (W/S ratio 1.6), carnosine (W/S ratio 2.5), and histidine (W/S ratio 1.4). In addition, proline levels increased 1.8-fold during winter; an amino acid that has not been quantified before. The aromatic amino acids tryptophan (W/S ratio 0.7) and tyrosine (W/S ratio 0.5), as well as sulfur-containing methionine (W/S ratio 0.7) and methionine sulfoxide (W/S ratio 0.2), had lower levels in hibernation. The latter is remarkable, as it may be taken as an indicator of reduced oxidative stress in hibernation. Our biogenic amine data are unique, and our amino acid data are in agreement with the metabolic changes reported by Stenvinkel et al. (18), and Sommer et al. (21) (Supplemental Table S2). Stenvinkel et al. (18) also reported significant 1.24-fold increases in total protein and albumin concentrations similar to our results (bottom of Supplemental Table S3). It is notable that our data and the available published nitrogen-related data, quantified by different methods, provide reciprocal validations.

Systemic Regulation—Sex-hormone-binding-globulin isoform 1 showed the highest increase in hibernation levels among all plasma proteins (SHGB, W/S ratio 45). This dramatic up-regulation was verified by western blot analysis, showing SHBG immuno-reactive bands in the plasma only from winter bears at ~47 and ~51 kDa (Fig. 1D). Human circulating and recombinant SHBG showed similar bands due to heterogeneous N-glycosylation forms (22). Our analysis of the human reference plasma sample did not reveal any SHGB immuno-reactive bands, suggesting that the SHGB levels in hibernating bears is markedly higher than the average human plasma levels (normal range 20-120 nM in adults). The very high SHBG levels in hibernating bears challenges the general understanding of SHBG's primary role as regulator of androgen bioavailability.

Seven other carriers of hydrophobic vitamins and hormones were down-regulated: beta-2-glycoprotein 1 (APOH, W/S ratio 0.69), alpha-1-acid-glycoprotein 2 (ORM2, W/S ratio 0.63), afamin (AFM, W/S ratio 0.87, p = 0.07), vitamin D-binding-protein isoform 3 (GC, W/S ratio 0.89, p = 0.16), retinol-binding-protein 4 (RBP4, W/S ratio
corticosteroid-binding-globulin (SERPINA6, W/S ratio 0.79), and transthyretin or thyroid-hormone-binding-protein (TTR, W/S ratio 0.67). Also thyroglobulin (TG), the precursor of thyroid hormones, was decreased in winter to 0.15-fold its summer levels. This observation is consistent with the lower levels of free thyroxine and triiodothyronine observed in hibernating captive black bear *Ursus americanus* and may, at least in part, explain the general lowered basal metabolism that characterizes hibernation (23).

Remarkably, the levels of the lipid mobilization factors adiponectin (ADIPOQ, W/S ratio 0.58) and zinc-alpha-glycoprotein (AZGP1, which is present at high levels in cancer cachexia and low in people with obesity; W/S ratio 0.25) were significantly decreased in hibernating bears, suggesting that bears utilize other systemic mediators of lipid mobilization during hibernation.

The decrease in the levels of the insulin-like-growth-factor-binding-protein-complex-acid-labile-subunit (IGFALS, W/S ratio 0.15) suggests a significant down-regulation of major circulating components of the IGF system in hibernating free-ranging brown bears, as also observed in captive American black bears (24). IGFALS is circulating in complex with IGF-1 and IGF-binding-protein 3 neither of which were identified, possibly due to very few detectable tryptic peptides of these proteins.

**Discussion**

We compared the levels of 154 plasma proteins covering four orders of magnitude of molar concentration, quantified 144 metabolites and blood cells in the same seven subadult free-ranging brown bears in winter hibernation and summer activity, and discovered a number of specific adaptations to hibernation involving defense against pathogens, lipid transport and metabolism, iron and nitrogen conservation, total protein levels and half-lives. The changes observed suggested a hibernation survival strategy using protective proteins with antimicrobial, clean-up and anti-oxidation properties, and conservation of energy in hibernation by down-regulating complex multiprotein cascades and up-regulating a small number of generalist and principal effector proteins, such as one lipase (CELL) and one protease inhibitor (A2M), both of broad-specificity.

The unique regulation of coagulation factors were verified by functional measurements (Table 1; Fig. 2). SHBG increased 45-fold in hibernation and appears to play a significant role in the maintenance of hibernation physiology. It is also notable that intracellular components from leaking or broken fat, muscle, bone, or liver cells were not detected. The biochemical data have been interpreted in the context of known physiological adaptations.

**Blood Characteristics**—Red bloods cell count, total hemoglobin levels, and the hematocrit were increased to 1.3-fold of the summer levels in hibernation. The summer levels are in agreement with recently published brown bear reference data (25). Total plasma protein concentration in hibernation was significantly increased by 9.6% in the seven 2010 samples, and by 24.1% in seven 2013 samples (Fig. 1A, Supplemental Table S5). The high hibernation levels of total protein were similar among all bears and similar to the normal human levels, whereas the lower summer levels showed considerable variance, because blood plasma concentration depends on the changing water content of available summer forage. ALB, which composes ~50% of plasma protein, was increased to 1.18-fold its summer levels (Supplemental Table S1). Stenvinkel et al. (18), using different methods, reported increases in total protein and ALB levels to 1.25-fold and 1.24-fold, respectively, in plasma samples from 16 hibernating young brown bears. These data explain both the lower yield and higher viscosity of plasma obtained from hibernating brown bears. The increased hematocrit to 1.3-fold of summer levels in hibernation is probably the best measure of dehydration.

**Defenses**—Of 36 proteins with defense properties that were measured by mass spectrometry in this study, 32 were significantly decreased in hibernation, including those of the lectin, alternative, and antibody complement pathways, the membrane pore-forming complex, and regulatory proteins including CRP (Fig. 2). In contrast, the levels of four protective antimicrobial proteins, CAMP, HP, LYZ, and PGLYRP2, and the antibody that is found in body secretions, IGA1, were more than doubled in hibernation (Supplemental Table S1). Hence, the hibernating bear can maintain solid first-line defense by *de novo* synthesis of just a few generalist proteins and a
secreted antibody rather than maintaining complex multiprotein pathways, thus saving energy.

HP, besides being antimicrobial, antioxidant and a recycler of iron (20), is most known as the kidney-protecting scavenger of hemoglobin released from bruised blood cells. Winter up-regulated APCS have similar protective scavenging properties.

Among these defense proteins, only the increases in HP hibernation levels have been documented previously (2- to 6-fold) (26). Besides HP, this immunochemical study of plasma collected samples at successive months during one year from captive brown bears included A2M (increased in hibernation), SERPINA1 (unchanged), and CRP (unchanged), and showed the same trends as our data (Supplemental Table S1). A comparative study of hibernating versus pre-hibernating captive female American black bear sera found 4-fold increases in HP levels (27). However, among the 15 proteins described in this two-dimensional gel electrophoresis study, our data showed pronounced disagreement with five of the reported changes. We suggest that the disagreement is due to the experimental design of Chow et al. (27), rather than the investigated bear species.

The innate and acquired cellular and humoral immune defenses were decreased, not abolished, in hibernation. As illustrated in Fig. 2, our data covers most components of the complement system and consistently demonstrated similar levels of reduction, including subunit levels of C1 and of the MAC complexes. Moreover, the complement regulatory proteins were reduced similarly to their targets. Our study is the first to distinguish the complement pathways of activation.

Coagulation and fibrinolysis must be strictly regulated to avoid thrombosis and pulmonary embolism. It appears that most specialized effector and regulator proteins of these pathways decreased in hibernation and were decreased in a balanced way as in the complement cascades. Yet hemostasis was maintained during hibernation increasing only the levels of the key effectors F10, F2 and fibrinogen, and the non-specific regulator A2M (Fig. 2). A recent study in 10 wild American black bears demonstrated that normal wound healing was maintained in hibernation, despite reduced skin temperatures, which were 4-6 °C lower than the hibernation body temperature (28). The first step in healing includes initial coagulation, which we showed is geared up by the slight increased levels of F10, F2 and fibrinogen, which will facilitate rapid initial response. Furthermore, our demonstrated increases in antimicrobial protein levels could indicate protection against wound infection, and might additionally substitute for inflammation, the second normal response in wound healing (28), as we found the levels of innate cells and components much reduced in hibernation.

The concentration of A2M, which has unspecific antiproteolytic activity, increased 1.7 fold in hibernation, whereas the specific SERPIN-type inhibitors, except for the anticoagulant SERPIN5, were significantly decreased in plasma (Fig. 2). Increased levels of A2M protein in hibernating brown bears was also found by Mominoki et al. (26); and Sheikh et al. (29) demonstrated similar increases in serum A2M from wild black bears using anti-bear A2M rabbit polyclonal antibodies. Most studies have applied anti-human antibodies, which in many cases have borderline cross-reactivity with bear proteins in our experience, due to limited amino acid sequence identity (Supplemental Table S1). Bear and human A2M are 81% identical in sequence and do cross-react, because of their large 180 kDa subunit sizes, which increase the probability of conserved immunogenic epitopes.

Intriguingly, Fuster et al. (30) reported a 40% decrease in protein degradation of isolated rat skeletal muscle incubated with plasma of hibernating brown bear versus controls without bear plasma. We propose the most likely inhibitor candidate is A2M. On proteolytic attack by any type of protease, tetrameric A2M changes its conformation due to activation of an internal thiol ester, and catches one molecule of the attacking protease thus inhibiting its activity. Therefore, we propose A2M might have a broad medical potential in reducing plasma protein degradation. Human A2M plasma concentrations are normally high (31), and vary considerably among individuals and with age (32). Hence, humans seem to tolerate increased A2M levels well, like bears in hibernation, which on average benefit from 1.7-fold increase in A2M levels.

Lipids—Some plasma proteins participating in lipid hydrolysis and transport were significantly up- or down-regulated in hibernation, which is concurrent with a switch from food ingestion to lipid oxidation. A hibernating 60 kg American black bear loses ~80 g fat per day (33). Adipose
tissue releases free fatty acids and glycerol, and the resulting shrinking of adipocyte membranes supported by dehydration are probably the main sources of increased phospholipid and cholesterol concentrations in the bloodstream during hibernation (Supplemental Tables S2, S3). The similar concentrations of sphingomyelins in winter and summer plasma were 2- to 5-fold greater than the concentrations in human plasma (Supplemental Table S2), and may protect bear cell membranes against degradation and rupture. This might explain the absence of intracellular components in our plasma data.

Chauhan et al. (34) separated serum cholesterols and some phospholipids by thin layer chromatography from four adult American female black bears, and found significant increases in free and esterified cholesterol, and in total SM, and total PC levels, which resemble the trends in our comprehensive data. Recently, Sommer et al. (21) analyzed metabolites by mass spectrometry in serum from 12-15 wild brown bears, and confirmed the presence of significantly increased levels of free cholesterol, cholesteryl esters, and triglycerides in hibernation, and also determined the levels of various bile salts, which decreased in hibernation. The study also found a general increase in hibernation in the levels of even chain acylcarnitines only. In contrast, we find a general increase in nearly all acylcarnitines (Supplemental Table S2), and our absolute concentrations are significantly higher than those reported by Sommer et al. (21).

Triglycerides are synthesized in the liver after uptake of free fatty acids and glycerol and provide energy to other tissues. The concentration of most lipids in plasma increased or was unchanged in hibernation (Supplemental Table S2). Major transporter proteins, ALB (which transports free fatty acids), APOB (which transports cholesterol, triglycerides and phospholipids in LDL and VLDL particles), as well as the predominant broad-specificity CEL lipase (increased by 32-fold), and total lipase activity were significantly up-regulated in hibernation. In contrast, the levels of a number of proteins interacting with HDL particles were decreased. APOA4 was extremely reduced to 0.04 times its summer levels, which is consistent with the function of the protein in signaling intestinal lipid uptake. The observations that only broad-specificity carriers of multiple lipid classes, APOB and ALB, and the generalist CEL lipase were increased in hibernation support our hypothesis of a common strategy of keeping generalist proteins at increased or normal activity levels, and let more specific ones decrease in hibernation.

Arinell et al. (14) reported increases in the levels of total cholesterol, LDL and triglycerides in hibernating brown bears and showed that none of 12 examined bears exhibited signs of atherosclerosis, unlike humans with similarly elevated plasma lipid levels. Our data support this important finding. We found 1.3-fold higher levels of APOB, the core protein in LDL and VLDL particles, in winter than in summer.

Nitrogen Metabolism and Protein Half-Lives in Hibernation—Absence of urination and the recycling of amino acids for protein synthesis during hibernation require that nitrogen be retained in a non-toxic form. Our analysis of amino acids and biogenic amines disclosed increased levels of molecules containing two or more nitrogen atoms (Supplemental Table S2), a strategy that has not been noticed before.

Intracellular proteins have short half-lives, minutes to days, whereas circulating ALB and IGG can last for months in humans (35). In hibernating bears all plasma protein half-lives will increase markedly, and reduce the need for costly protein synthesis for three reasons. (i) The increased levels of A2M, discussed above, will decrease protein turn-over by reducing all protease activity. (ii) Reaction rates, including protein degradation, will decrease with temperature. A generalization of Arrhenius' equation implies that the rates of chemical reactions at room temperature will double for every 10 °C increase in temperature. The temperature in hibernating non-pregnant bears is lowered by 6 °C on average (5-7). (iii) Cardiac output in free-ranging hibernating brown bears is reduced to 24% from 3.5 to 0.86 L min⁻¹ (8), i.e. similar to the 25% level of oxygen consumption or metabolic rate in hibernating American black bear (5). Jørgensen et al. (8) also observed echo-dense structures in the hearts of all the hibernating bears caused by aggregation of red blood cells and plasma proteins. Therefore, rates of productive encounters of biochemical partners will decrease, due to reduced mixing rates in blood, as well as increased plasma viscosity and, consequently, decrease protein modification rates and turn-over in hibernation.
Protein Levels and Energy Conservation—The increased hematocrit in hibernation demonstrates significant dehydration. In hibernating bears water comes from fat metabolism exclusively. The complete reaction of 1 mol of triglyceride (0.89 kg tristearylglycerol C₃₇H₁₁₀O₆) and oxygen yields 55 mol of water, 0.99 kg or 1 L. Since ~80 g fat is burned per day, it takes 11 days to produce 1 L water. Water loss is mainly through the breathing of saturated water vapor at the body temperature of hibernation at ~32 ºC, whereas the air in the den at subzero temperatures gives little water vapor back. We conclude that dehydration is substantial and contributes to the maintenance of plasma protein concentrations in hibernation, thereby saving much novel protein synthesis and energy. Moreover, ALB constitutes ~50% of total plasma protein and has a very long half-life, which alone contributes to significant energy savings in hibernation.

Energy conservation is a key issue in all organisms and vital in hibernation. Protein synthesis at unstressed conditions consumes more than half of organismal energy. Furthermore, it can be strictly regulated for energy savings in various ways. Li et al. (36) quantified absolute protein synthesis rates by deep sequencing of ribosome-protected mRNA fragments in E. coli grown on limited and rich media. They concluded that subunits of multiprotein complexes are synthesized in equal proportion regulated by translation initiation and that this rule also applies to yeast, whereas protein synthesis in multicellular organisms might be fine-tuned by additional rules. Pannevis and Houlihan (37) found that isolated hepatocytes of rainbow trout used 70-90% of their total oxygen consumption for cycloheximide-sensitive protein synthesis, and that the energy costs were lowered at decreased rates of protein synthesis, i.e. subject to regulation. This relates to hibernating bears, as liver is the principal tissue for plasma protein synthesis, and the rate of protein synthesis is low.

Schwanhäusser et al. (38) quantified mRNA and protein levels, and their half-lives globally in pulse labelled mouse fibroblast cultures, and derived a model predicting protein abundance in cells. Genes with stable mRNAs and stable proteins were enriched in constitutive cellular processes maintaining basic metabolism, suggesting an evolutionary pressure for energy saving. Moreover, they found that protein synthesis consumes more than 90% and transcription less than 10% of cell energy. Thus it is plausible that bears can save expensive translational energy and be easily aroused, just by having increased levels of mRNA ready for the needed proteins and the translational machinery, at a low energy cost. Indeed, Fedorov et al. (11, 12) in micro array studies of gene expression in American black bear liver, heart, and muscle tissues showed a marked up-regulation of mRNA from genes participating in translation in these tissues. Liver also showed up-regulation of fatty acid beta-oxidation in hibernation as expected. In contrast, amino acid catabolism, cholesterol metabolism, and cellular respiration were significantly down-regulated in hibernation.

In hibernating American black bears protein synthesis and break-down were balanced in skeletal muscle and were 60-70% decreased in winter compared to summer (10). Thus even maintaining the levels of all muscle proteins lead to energy savings approaching the required 75% in hibernation. Therefore, we conclude that decreased levels of the majority of blood plasma proteins and the maintenance or increase of levels of just a few key proteins in blood plasma by liver protein synthesis is the principal energy saver in bear hibernation adaptation, due to dehydration, a lowered temperature, a general down-regulation of translation, and a balanced translation of effector and regulator proteins in the complement and blood clotting pathways.

Systemic Regulation—It remains obscure how hibernation is initiated, maintained and completed, although the 45-fold increased levels of SHBG in hibernation (confirmed by immunoblotting Fig. 1D) provides a clue to the maintenance of hibernation physiology. SHBG is generally considered the primary regulator of androgen bioavailability, as seen in the seasonal changes in SHBG concentration in the hibernating little brown bat, having low baseline SHBG levels during the October-April hibernation period, and dramatically peaking during the sexually active June-July period (39). In contrast, testosterone levels in American black bears are high March through mid-July, and low mid-July through December, independent of nutritional and social factors (40). Palmer et al. (41) confirmed and extended the black bear testosterone data, which indicated 2-fold higher levels in January-March than in July-November. We suggest this difference may compensate for hormones
bound to the high levels of SHBP in this condition, thus keeping free hormone levels constant. The hibernating European hedgehog appears to be more similar to bear with increased SHBG levels in September-January with a sharp decline in February, reverse of testosterone levels (42). We conclude that bear SHBG primarily acts sex hormone-independently and can activate the SHBG receptor directly. In target cells the SHBG receptor increases cAMP production, which activates protein kinase A (43), thus activating signaling pathways different from sex hormone activated pathways. In fact, high circulating SHBG levels in humans protect against metabolic syndrome, and increases insulin sensitivity independent of sex-hormone concentrations (44). In addition, high SHBG levels have been found to correlate with lower cardiovascular disease risk factors in young men (45). Recently, SHBG deficiency was found in a man suffering from muscle weakness and fatigue, suggesting a direct impact of SHBG also on muscle maintenance (46). We conclude that high SHBP levels appear to be beneficial to health via presently unknown mechanisms.

Limitations and Prospects—Quantification of signaling proteins and hormones present at a very low concentration or of small size was outside the capability of our methodology. The bear protein database was effective, but had shortcomings, with some expected proteins missing and others with inaccurate translation.

We have determined and interpreted the changed levels of blood and plasma constituents in free-ranging subadult brown bears on a global scale in winter and summer with emphasis on conservation of health and energy in hibernation. Our results reveal a number of unexpected changes in protein concentration in hibernation, some expanding fundamental physiologic understanding derived from human and non-hibernating animal models. Study of the function and regulation of these proteins and their genes, in the light of bear physiology, will add to and refine present interpretations of complex mammalian biochemical networks and might encourage the development of novel clinical therapies.

Experimental Procedures

Animals—The Scandinavian Brown Bear Research Project (http://www.bearproject.info) collects blood samples twice a year from free-ranging (wild) bears in Dalarna, Central Sweden, during hibernation and activity. All captures were approved by the Swedish Ethical Committee on Animal Research (application numbers C212/9 and C47/9) and the Swedish Environmental Protection Agency. Bears were fitted with GPS collars and VHF transmitters on their first capture at one year of age, when still with the mother. The collared bears were immobilized from a helicopter in summer by a combination of tiletamine-zolazepam and medetomidine, and in the dens in winter giving also ketamine. Anesthesia was antagonized by atipamezol, and the hibernating bears were placed back into the dens. Details on the methods of bear capture, anesthesia and blood sample collection have been published previously (16). Seven subadult bears were analyzed in the present study, B1 through B7 (alias bear ID 0824M, 0910M, 0825F, 0820F, 0819F, 0818F, and 0812M, respectively), comprised four 3-year old nulliparous females, and two 3-year old and one 2-year old males.

Blood and Plasma Samples—Arterial blood was collected in EDTA-tubes (K2E 10.8 mg, 6 mL, BD ref 365900, Becton, Dickinson and Company, NJ USA) during hibernation in February 2010 and again from the same animals when active in June 2010. The blood was kept on ice until centrifugation at 200 g for 15 min, immediately upon returning from the field after 1-2 h. The yield of plasma from winter blood was notably lower. Plasma was frozen and kept on dry ice during shipping. Plasma samples were thawed only once, flash frozen in ca. 25 µL pearls in liquid nitrogen, and stored at -80 °C. A pooled normal human plasma reference sample, K2 EDTA lot 23-40651A (Innovative Research, Mi USA), was treated similarly. Similar paired samples collected in 2012 and 2013 were used for specific validation tests.

Hematology—Blood cell counts and standard biochemical analyses were carried out at the accredited Clinical Chemical Laboratory at Örebro University Hospital, Sweden, as described previously (6, 14). Blood cell analyses were carried out by an automated hematology analyzer (XE-5000, Sysmex Corporation, Kobe Japan); and biochemical analyses in the integrated Vitros instrument (Ortho-Clinical Diagnostics, Rochester, NY USA). Coagulation analyses were carried out at the accredited Clinical Chemical Laboratory at
Linköping University Hospital, Sweden, utilizing an automated coagulation analyzer ACL TOP (Instrumentation Laboratory, Bedford, Massachusetts, USA). Factors F2 and F7 were determined using a one-stage clotting assay with deficiency plasmas (Precision BioLogic, Dartmouth, Canada); Fibrinogen was measured by the Clauss method determining the time of clot formation; F8 and PROC were measured by proteolytic cleavage of specific chromogenic peptides, and SERPINC1 indirectly by inhibition of F10 activity in a chromogenic assay. VWF was quantified in an immunoassay.

Immunoblotting—Plasma (0.25 µL) was subjected to 4-20% reducing SDS-PAGE. A precasted protein ladder 10-170 kDa (0.5 µL) was included (SM0671, Fermentas). Proteins were blotted to a nitrocellulose membrane and incubated in sequence with 2 mL rabbit polyclonal anti-SHBG primary antibody (ab119436; a 100 residues linear human polypeptide, with segments identical in amino acid sequence to bear and different from rabbit SHBG, was used as immunogen), Abcam, Cambridge UK) in TBS-1% BSA (1:200), and 2 mL donkey anti-rabbit/Alexa Fluor 647 conjugated secondary antibody (ab150075) in TBS-0.1% Tween20 (1:2000) applying TBS-Tween20 washes in an iBind Western System (Life Technologies). Proteins were visualized in a ChemiDoc MP imaging system (Bio-Rad, CA USA).

Plasma Protein and Hemoglobin Concentration—Ultraviolet and ultraviolet-visible absorption spectra were recorded directly on 2 µL undiluted plasma samples using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, De USA). The instrument automatically used a 0.2 mm path length for protein measurement at 220-350 nm and reported the values for 1 cm path length. Owing to a visible content of hemoglobin, the automatic normalizing A340 nm = 0 was turned off. Spectral data were transferred to an Excel spread sheet (Microsoft Office 2013) and normalized to identical A340 nm values. A280 nm was then used as a measure of protein concentration in g L⁻¹, however corrected by our estimated E0.15 cm⁻¹ = 0.87 for bear plasma (Table S5).

Hemoglobin (HB) spectra were recorded at 220-750 nm at 1 mm light path length. The spectra were normalized in Excel by setting the measured mean values 650-750 nm = 0 and adjusting to 1 cm path length. The spectra showed that HB was fully oxygenated with the Soret peak at 415 nm. The extinction coefficient for tetrameric human oxyhemoglobin at 415 nm, 524280 M⁻¹cm⁻¹, was applied.

Protein Digestion and Peptide Desalting—For proteomic analyses the tryptic digestion, peptide desalting and MS analyses were performed in two batches of samples. Batch 1 included winter and summer pairs of bear samples B1, B2, a sample of recombinant human insulin-growth-factor-like (IGF1) in complex with IGF-binding-protein 3 paired with a water blank, and winter and summer pairs of bear samples B3, B4, B5, B6, and B7, i.e. a total of 16 samples run in the sequence shown, each measured in three consecutive technical replicates. Batch 2 included the commercially available human plasma reference samples (Hs) paired by itself, the B2 pair for a second time (which was due to first round MS instrument failure), and ProteoMiner-enriched bear mixed plasma samples pairs from winter and summer, i.e. six samples prepared and measured in parallel each in three technical replicates.

Protein was digested with trypsin essentially as described by Wiśniewski et al. (47). Plasma (3 µL) protein was unfolded and reduced at 95 °C for 10 min after mixing with 4% SDS, 100 mM DTT, 5 mM EDTA in 0.1 M TrisCl pH 8.5 (17 µL). A volume containing 100 µg protein was transferred to the filter unit (reactor) of a 30 k Amicon ultra cell containing 200 µL 8 M urea in 0.1 M TrisCl pH 8.5. Centrifugations between steps were carried out at 13,500 g for 15 min. An extra wash was carried out before alkylation, which was carried out in the same solvent containing 25 mM iodoacetic acid for 20 min at room temperature. After three washes using 100 µL 8 M urea in 0.1 M TrisCl pH 8.0, urea concentration was reduced by washing with 200 µL 2 M urea in 0.1 M TrisCl pH 8.0. Collection tubes below the filter reactors were replaced with clean ones before sequencing-grade modified porcine trypsin (Promega, Wi USA) 1 µg in 2 µL and then 50 µL 2 M urea in 0.1 M TrisCl pH 8.0 were added to the filter reactor and mixed by tapping. Digestion at room temperature was continued for 20 h. Peptides were spun down into the collection tube and further washed down by 60 µL 2 M urea in 0.1 M TrisCl pH 8.0, and at last by 200 µL water. Peptides were adsorbed to 1 mg washed R3 Poros beads (Applied Biosystems, Ca USA) in 20 µL suspension of 5% formic acid added to the
collection tube, together with 5 pmol human [Glu1]-Fibrinopeptide B for internal MS standard (Sigma-Aldrich, Mi USA) just before the final peptide extraction by water. Beads were collected by centrifugation at 4,000 g for 1 min and washed twice with 200 µL 1% formic acid. Supernatants were removed carefully by micro pipetting. Beads with bound peptides were stored in 50 µL A solvent (0.1% trifluoroacetic acid in 2% acetonitrile) at 4 °C. The pH values were confirmed at all critical steps by applying a fine drop on pH paper.

Columns of 200 µL pipette tips were plugged with a disc of polystyrene-divinylbenzene (Empore SDB-XC; 3M company, Mn USA) punched out by a metal syringe needle and washed with 5 µL B solvent (0.1% trifluoroacetic acid in 90% acetonitrile) followed by 10 µL A solvent. A volume of suspended R3 beads with peptides were transferred to the column and allowed to drain, then washed with 20 µL A solvent, and eluted into an MS-sample plate, twice with 5 µL B solvent and finally with 10 µL A solvent to elute remaining dissolved peptides. The acetonitrile content was reduced by evaporation, and the wet peptide sample (approximately 25 µg) was diluted by 30 µL 0.1% formic acid, 0.005% heptafluorobutyric acid.

Test experiments prior to final digestions had protein and peptide yields monitored by UV absorption spectroscopy (NanoDrop) as above. Peptide yield was > 50% of original protein content. Initial peptide binding by C18 filters for desalting (3M, Mn USA) was insufficient in our hands. Comparison of adsorption and release of peptides by R2 versus R3 Poros beads demonstrated a higher binding capacity and complete elution from the R3 beads.

LC-MS/MS Data Acquisition—The LC-MS/MS analysis was done using a Dionex Ultimate 3000 nanoLC system connected to a quadrupole Orbitrap (Q Exactive) mass spectrometer equipped with a NanoSpray Flex ion source (Thermo Fisher Scientific, Ma USA). The LC solvents were A: 0.1% formic acid, 0.005% heptafluorobutyric acid for the aqueous phase, and B: 90% acetonitrile, 0.1% formic acid, 0.005% heptafluorobutyric acid for the organic phase. The applied gradient was from 12% to 20% solvent B over 60 min, followed by 20% to 40% solvent B over 60 min. Initial tests showed that this gradient gave the best distribution of plasma protein tryptic peptides over the entire gradient.

The Q Exactive mass spectrometer was operated in a data-dependent acquisition mode. A full MS scan in the mass range of 350-1,850 m/z was acquired at a resolution of 70,000 with an automatic gain control (AGC) target of 3×10^6 and maximum fill time set to 250 ms. Instrument lock mass correction was applied using the contaminant ion at 391.28429 m/z. In each cycle, the mass spectrometer triggers up to 12 MS/MS acquisitions on abundant peptide precursor ions. The MS/MS scans were acquired with a dynamic mass range at a resolution of 17,500, and with an AGC target of 2×10^5 and maximum fill time of 60 ms. The precursor ions were isolated using a quadrupole isolation window of 3 m/z, and then fragmented in the higher-energy collisional dissociation (HCD) trap with a normalized collision energy set to 30. The under-fill ratio was set to 3.5% with the intensity threshold at 1.2×10^5. Apex triggering was 3 s to 10 s with charge and exclude isotopes exclusion on, and dynamic exclusion set to 30 s. The mass spectrometry proteomics data are available via ProteomeXchange with identifier PXD003946 at http://www.ebi.ac.uk/pride.

Quantitative Data Analysis Using Progenesis LC-MS Software—Three technical replicates for each plasma sample were generated using LC-MS/MS as described above. The six raw data files (winter and summer) for each bear and for controls were analyzed separately in Progenesis LC-MS v4.1 (Nonlinear Dynamics, Newcastle Upon Tyne, UK). The raw file features were automatically aligned to an assigned reference file among the six raw files, and peak picking was also done in automatic mode. Peak filtering was carried out with respect to charge state (up to 7 charges) and retention time features within 30 min to 140 min were kept. Protein identifications were obtained using Mascot Server v2.3 (Matrix Sciences, London UK). The database search parameters were precursor and fragment tolerances of 6 ppm and 20 ppm, respectively. One missed cleavage was allowed, cysteine
carboxymethylation was fixed, whereas methionine oxidation was set as a variable. False discovery rates (FDR) were 0.3-0.95% with an average of 0.71%. The protein database (Uma db, Supplemental Bear-protein-db-Febr2013.txt) was translated from predicted polar bear Ursus maritimus coding sequences (17). The genome sequences of polar bear and brown bear are similar, showing, on average, two substitutions per 1,000 bp (48). 93% could be annotated by homology using a BLASTP search against the human refseq database (E-value ≤ 1×10⁻⁵). This Uma db contained 21,143 entries; 42% showed ≥ 90% identity to the human homolog. The Uma db yielded approximately 12% more identified proteins than our initial db based on a combination of translated giant panda Ailuropoda melanoleuca genes and American black bear Ursus americanus EST sequences (49, 50).

The Progenesis LC-MS data output was an Excel sheet containing normalized ion counts (summed using unique peptides) for each protein (Supplemental Table S5). All data from the seven bears were merged using the mean values of the three technical replicates for each sample. The data was then scaled to normalize for the differences in the Progenesis LC-MS output for each bear. The normalized mean values were used to calculate W/S ratios (fold changes) and p-values (paired t-test). W/S ratios of proteins identified by two unique peptides and seen in at least four of seven bears were saved in Proteins.xlsx (Supplemental Table S1) using the gene names of human homologs, together with their Uma and Uniprot ID, fold change and log2 fold change, paired t-test p-values, and Uniprot function descriptions. Principal component analysis and Volcano plots were done in R version 3.1.1 using the ggplot2 package (51, 52).

Identification and Quantification of Metabolites—The same seven paired sets of winter and summer bear, and the human reference plasma samples were subjected to metabolite concentration determination by the AbsoluteIDQ p180 kit (Biocrates Life Sciences, Innsbruck, Austria). Nineteen biogenic amines, 19 amino acids, 1 hexose, 40 acylcarnitines, 76 phosphatidylcholines, 14 lysophosphatidylcholines, and 15 sphingomyelins were analyzed in a 96-well plate format essentially as described by the manufacturer. Each plasma sample (10 µL) was treated with phenylisothiocyanate to modify amines. Analytes were quantified relative to internal standards of isotopic labelled homologs by multi reaction monitoring QTRAP mass spectrometry and electrospray ionization using an ABI ScieX API5500Q-TRAP instrument (AB SCIEX, Ma USA). Each sample was subjected to LC-MS analyses of biogenic amines and amino acids, and to flow injection analysis-MS/MS of the remainder. Both measurements were performed in duplicate and in positive and negative ionization mode. The dedicated MetIDQ software (Biocrates Life Sciences) provided automated calculation of metabolite concentrations.

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Hibernation Survival Strategies in Brown Bear

This article contains Supplemental Tables S1 to S5, and the database Bear-protein-db-Febr2013.txt.

References
Hibernation Survival Strategies in Brown Bear


Figure Legends

FIGURE 1. Protein and metabolite changes in blood plasma in free-ranging brown bears during hibernation in winter and in the same bears when active in summer. (A) Absorption spectra of undiluted plasma samples collected in winter (W) and summer (S) from seven brown bears (bear IDs at top right. M, male; F, female. All were 3-year old, except for the 2-year old 0910M). Top: Total protein at 280 nm; bottom: Hemoglobin at 415 nm (Supplemental Table S5). (B) Principal component analyses of plasma proteins, amines and phospholipids showed a clear separation of winter (red) and summer (blue) plasma. (C) Volcano plots of W/S ratios of plasma proteins, amines and phospholipids versus p-values from paired t-tests. Dashed lines at p = 0.05. (D) Immunoblot of sex-hormone-binding-globulin (SHBG) confirming the large increase in hibernation [proteomics detected 45-fold increased levels in winter plasma (p = 0.0006)]. The arrows indicate two forms of glycosylated SHBG [like in human SHBG (28)]. Heavy and light chains of bear IgG were ~52 kDa and 27 kDa. All seven bears analyzed in 2010 showed identical results, which were confirmed by samples collected from subadult bears in 2012 and 2013.

FIGURE 2. Interplay and regulation of plasma protein factors in coagulation, fibrinolysis and complement defense in hibernating brown bears. Protein names come from the corresponding human genes. The mean of winter/summer (W/S) ratios of protein levels for each of seven subadult bears and the p-values from paired t-tests quantified by mass spectrometry have been summarized and illustrated by squared frames. Thick frame, p-value < 0.05. W/S ratio increased to > 1.2-fold in hibernation (red); Moderate W/S ratio changes 0.8 to 1.2-fold (black); W/S ratio decreased to < 0.8-fold in hibernation (blue). Component not detected (dashed). Component absent in the bear protein database (yellow). Coagulation factors quantified by functional assays are shown in space filling color, red increased and blue decreased in hibernation (Table 1). The figure was modified after http://www.reactome.org/PathwayBrowser/.
Fig. 1
Hibernation Survival Strategies in Brown Bear

Coagulation cascade
Extrinsic pathway:
Intrinsic pathway:

Kallikrein-kinin system

Complement cascade
Alternative pathway:
Lectin pathway:
Classical pathway:

Fig. 2